Genome-wide characterisation of microRNAs and their target genes in different durum wheat genotypes under water limiting conditions

By

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Abstract

Durum wheat (*Triticum turgidum* L. ssp. *durum*) is a tetraploid wheat species grown primarily in the North American Great Plains, Mediterranean Europe, Northern Africa, Mexico and Australia. An important limiting factor for durum production in Mediterranean environments like South Australia is water deficit immediately prior to and during anthesis, adversely affecting durum productivity and quality. Investigating water deficit response mechanisms and genotypic differences within a crop species is an important strategy for understanding the basis of water-deficit stress response and for selection of elite genotypes with improved stress tolerance. In plants, microRNAs (miRNAs), which are a class of small non coding RNAs, have been identified as important regulators of plant development and abiotic stress responses. While the miRNA transcriptome under water limiting conditions has been investigated in many crop species, it is poorly characterised in durum wheat.

In this study, glasshouse experiments over two years evaluated 20 durum wheat genotypes for their variation in various morphological, physiological and yield responses to pre-anthesis water-deficit stress. Four Australian durum varieties with contrasting stress sensitivities were identified. High-throughput Illumina sequencing of 96 small RNA libraries constructed from the flag leaf and head tissues of these four genotypes detected 110 conserved miRNAs and 159 novel candidate miRNA hairpins. Statistical analysis of sequencing reads revealed the differential expression profiles of durum miRNAs associated with water-deficit stress treatment, tissue type and genotype. Most importantly, several conserved and novel miRNAs showed inverted regulatory profiles between the stress tolerant and sensitive varieties. Subsequent genome-wide *in silico* analysis identified 2055 putative targets for conserved durum miRNAs, and 131 targets for four novel durum miRNAs possibly contributing to genotypic stress tolerance. Predicted mRNA targets of the stress responsive miRNAs encode various transcription factors, binding proteins, and functional enzymes,

which play vital roles in multiple biological pathways such as hormone signalling and metabolic processes, suggesting the extensive involvement of miRNA-target regulatory modules in water-deficit stress adaptation. Quantitative PCR profiling further characterised 50 target genes and 12 miRNAs with stress responsive and/or genotype-dependent expression profiles. A 5′ RLM-RACE approach subsequently validated the regulation of nine targets by water-deficit stress responsive miRNAs, providing the first experimental evidence that target mRNAs are genuinely cleaved by miRNAs in durum wheat. Characterisation of the individual miR160/Auxin Response Factors regulatory module further revealed their expression profile over different time points during water-deficit stress.

The present study provides a comprehensive and comparative description of the miRNA transcriptome and their targets in durum wheat varieties with contrasting water-deficit stress tolerance, providing new insights into the functional roles of miRNA-guided RNAi mechanisms. Results derived from this work could contribute to future research on the characterisation of individual miRNA regulatory modules and their specific biological functions, exploiting the potential of *Triticum turgidum* miRNA in developing RNAi-improved crops with stress tolerance.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Chapter 1

Preface

Chapter 1: Introduction to durum wheat, water-deficit stress and small RNAs

1.1 Significance of durum wheat and production challenges

Durum wheat (*Triticum turgidum* L. ssp. *durum*) is a monocotyledonous cereal species from the genus *Triticum* in the *Triticeae* tribe. It originated in the Eastern Mediterranean through intergeneric hybridisation and polyploidisation involving two diploid grass species, *Triticum urartu* (Dvorak 1976) and *Aegilops speltoides* (Riley *et al.* 1958). Durum is a tetraploid wheat species (2n = 4x = 28, genomes AABB) grown commercially because of its unique grain characteristics and versatile end uses. Currently, durum wheat is primarily cultivated in the North American Great Plains, Mediterranean Europe, Northern Africa, Mexico and Australia (Leff *et al.* 2004; Habash *et al.* 2009; Ren *et al.* 2013). Durum wheat grain is typically large and translucent, with a higher yellow pigment and protein content when compared to bread wheat (*Triticum aestivum* L.) (Li *et al.* 2013). Commonly considered as the hardiest wheat with inextensible gluten, durum wheat can be used for various food products including pasta, couscous, flat bread, bulgur and freekeh.

During the past decade, annual global production of durum wheat fluctuated between 33 and 41 million tonnes (International Grains Council 2016). This variation in production can largely be attributed to various abiotic stress constraints, including drought and temperature extremes, which occur frequently in the natural rain-fed environments of the SEWANA region (South Europe, West Asia and North Africa) (Li *et al.* 2013; Longin *et al.* 2013). Breeding for water-deficit stress tolerance has therefore become a major objective for durum breeders not only in these areas, but wherever drought and temperature extremes have occurred. In Australia, durum wheat is primarily grown in northern New South Wales, South Australia and western Victoria, and like the SEWANA region, one of the biggest constraints for improving durum

wheat production is the availability of water. Water deficiency, caused by the lack of rainfall and declining soil moisture during critical stages of crop development can be a common phenomenon across Australia's wheat belt but particularly in Southern Australia (French & Schultz 1984; Nicholls *et al.* 1997; Garcia del Moral *et al.* 2003; Liu *et al.* 2015a).

The occurrence of water-deficit stress during crucial periods of plant development such as flowering, pollination and grain-filling can lead to defective reproductive structures, which in turn will significantly reduce final grain yield (Yang *et al.* 2001; Foulkes *et al.* 2007; Habash *et al.* 2009; Katerji *et al.* 2009; Ji *et al.* 2010). In the main durum growing regions of Australia, most rainfall occurs in winter, and water deficit often appears in spring (Liu *et al.* 2015a). This leads to moderate water-deficit stress around the pre-anthesis stage, and the stress might intensify throughout flowering and grain filling. Studies of water deficiency that occur at post-anthesis stages have shown severe detrimental effects on grain size rather than grain number, due to the changes in the grain filling rate when the grain number is already established (Shah & Paulsen 2003; Plaut *et al.* 2004; Ercoli *et al.* 2008; Sanjari Pireivatlou & Yazdansepas 2010). Water-deficit stress at heading could reduce the number of grains per spike by increasing rates of spikelet abortion and pollen sterility (Praba *et al.* 2009; Sanjari Pireivatlou & Yazdansepas 2010). For durum wheat, there is limited literature on the effects of pre-anthesis water-deficit stress despite the significant effects it can have on crop yield. Given that precipitation can fluctuate significantly across Australia in any one year, understanding the mechanisms of stress response to pre-anthesis water deficiency in durum wheat, and breeding for elite varieties with improved tolerance, *albeit* challenging, are of great importance.

1.2 Improving water-deficit stress tolerance in durum wheat

To screen, select and develop elite varieties capable of tolerating water-deficit stress, an understanding of plant stress tolerance is essential. In general, plants could perceive, respond, and adapt to abiotic stresses at various morphological, physiological, biochemical and molecular levels. Different strategies could be involved in plant responses to water stress at these levels based on the framework developed by Levitt and include stress escape, stress avoidance and stress tolerance (Levitt 1980). Stress escape allows the crops to escape from unfavourable conditions with water deficiency, which is normally achieved by a shorter life cycle and developmental plasticity such as early flowering and maturity (Levitt 1980; Richards *et al.* 2002). Stress avoidance strategies decrease the cellular stress level through mostly morphological changes such as deeper roots to maximise soil water uptake (Levitt 1980; Yue *et al.* 2006). Finally, stress tolerance involves mainly physiological and biochemical responses that minimise the damage caused by stress, such as enhanced antioxidative activity and well-partitioned dry matter accumulation (Blum 2005; Simova-Stoilova *et al.* 2009). However, the key to the successful adoption of these stress response strategies is the balance between improving water use efficiency and maximising yield potential (Richards *et al.* 2002; Blum 2005; Tuberosa & Salvi 2006; Cattivelli *et al.* 2008). This requires more emphasis on the stress tolerance strategy, which determines the ability for crops to achieve acceptable yield under mild stress (Tuberosa & Salvi 2006; Tardieu & Tuberosa 2010), the ultimate goal of crop improvement in water limiting regions.

An important strategy adopted in cereal breeding to improve yield under stressful environments is to select target traits closely correlated with yield components and yield potential (Cattivelli *et al.* 2008; Habash *et al.* 2009). Studies on cereal crops under drought environments have identified several traits such as leaf water potential, chlorophyll content, photosynthetic rate, stomatal conductance, and transpiration rate (Li *et al.* 2006;

Subrahmanyam *et al.* 2006; Khanna-Chopra & Selote 2007; Arjenaki *et al.* 2012). However, many of the traits were characterised under field conditions where crops were exposed to varied and uncontrollable stress conditions. Moreover, crop breeders' efforts sometimes lead to the development of lines with stress avoidance, thus the effects of water deficiency are limited to later developmental stages. Controlled glasshouse experiments enable precise control of the variables of water-deficit stress (for example, timing, duration and level), thereby minimising confounding effects that could lead to results being misinterpreted. Thus, the correlation of the physiological and morphological traits between yield components under pre-anthesis water-deficit stress, and their natural genotypic differences among different durum wheat varieties could be investigated, which would assist breeders to develop a better understanding of stress tolerance in durum and identify adaptive genotypes under Mediterranean conditions.

Another strategy of great importance in improving stress tolerance is to identify and modulate the molecular regulatory pathways that underlie stress responses and adaptation. Crops exposed to abiotic stresses use complex yet well-coordinated mechanisms to reprogram molecular events that prompt adaptive changes at morphological and physiological levels to guarantee survival and reproductive success. Emerging modern techniques such as high-throughput sequencing, suppression subtractive hybridisation and cDNA/RNA microarray have contributed greatly to the identification of stress-inducible genes, signalling transporters, and epigenetic regulators governing stress tolerance in many crops (Wang *et al.* 2010; Deokar *et al.* 2011; Puranik *et al.* 2011; Seiler *et al.* 2011; Wang *et al.* 2011; Barrera-Figueroa *et al.* 2012; Budak *et al.* 2015b). The successful applications of some of these discoveries in the genetic improvement of other cereal species (Yang *et al.* 2013; Zhang *et al.* 2013; Chen *et al.* 2015; Gao *et al.* 2015) suggests that there is potential to explore such technologies in durum wheat. However, compared with closely-related bread wheat, durum wheat has received far less research attention. Investigating key molecular players that are involved in stress responses and

their natural genetic diversity in different durum genotypes would therefore be of significant benefit to breeding programs around the world. Ultimately, understanding and unlocking such molecular potential would enable the development of elite durum wheat varieties with improved tolerance and higher yield stability.

1.3 Molecular breeding and the potential of small RNAs

Small non-coding RNAs (sRNAs) of 20-24 nucleotides (nts) have emerged as master epigenetic regulators of gene expression during plant development and stress responses (Reinhart *et al.* 2002; Carrington & Ambros 2003; Jones-Rhoades & Bartel 2004; Bond & Baulcombe 2014; Hisanaga *et al.* 2014; Borges & Martienssen 2015; Wang & Chekanova 2016). Small RNAs can precisely reprogram the expression of stress- or development-associated genes through transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) (Xie *et al.* 2004; Borges & Martienssen 2015). Plant small RNAs can be classified into two main categories, microRNAs (miRNAs) and small interfering RNAs (siRNAs), distinguished by their biogenesis and function (Borges & Martienssen 2015). Generally, mature single-stranded miRNAs are processed from precursor miRNAs, which originate from hairpin primary-miRNAs transcribed from MIR genes. siRNAs are derived from long double-stranded RNA (dsRNA) precursors, which can originate from non-coding loci and protein-coding genes in the euchromatin or DNA repeats and transposons in the heterochromatin (Borges & Martienssen 2015). Mature miRNAs are loaded into the RNA-induced silencing complex (RISC) in association with Argonaute (AGO) proteins in the RNA silencing mechanism. Mature miRNA in the RISC control the expression of its target gene(s) by binding to the imperfect reverse complementary sequences within the cognate mRNA targets, inducing either cleavage degradation or translational inhibition (Jones-Rhoades *et al.* 2006; Sunkar *et al.* 2007; Borges & Martienssen 2015).

The extensive involvement of miRNAs and their functional target genes in various biological processes has been demonstrated in many plant species (Yang *et al.* 2013; Zhang *et al.* 2013; Peng *et al.* 2014; Akpinar *et al.* 2015; Budak *et al.* 2015a; Wang *et al.* 2015; Xie *et al.* 2015; Vialette-Guiraud *et al.* 2016). Most importantly, miRNAs can respond to and integrate both environmental and developmental cues, reprogramming numerous downstream gene transcription events so as to contribute to plant fitness and survival (Budak et al. 2015b; Sunkar et al. 2012; Wang and Chekanova 2016; Zhang 2015). Even subtle and transient changes in the miRNA expression level during stress could lead to profound physiological and morphological effects (Sunkar *et al.* 2012; Ding *et al.* 2013; Zhang 2015). To explore the potential of miRNAs in stress tolerance improvement, a number of studies have already been conducted in cereal crops to identify stress-associated miRNAs and their functional targets (Kantar *et al.* 2010; Budak & Akpinar 2011; Kantar *et al.* 2011; Yang *et al.* 2013; Han *et al.* 2014; Akpinar *et al.* 2015; Budak *et al.* 2015a; Cheah *et al.* 2015; Li *et al.* 2015). However, very little is known about durum miRNAs and their regulatory roles in water stress responses among different durum genotypes.

1.4 Objectives and main achievements of this study

The focus of the review article published in *Trends in Plant Science* [Chapter 2 (Liu *et al.* 2016a)] centres on cereal breeding and the application of miRNAs, the current status of wheat miRNA modules and their specific regulatory roles in stress response and development. Thus it is sufficiently similar to the overall objectives of this study to be used as the literature review.

From a research perspective this study had three main objectives. The first objective was to assess the morphological and physiological responses of 20 durum wheat genotypes

exposed to pre-anthesis water-deficit stress [Chapter 3 (Liu *et al.* 2015a) *Crop & Pasture Science*]. To accomplish this, these genotypes were evaluated in glasshouse experiments across two-years, which enabled the identification of target traits that could possibly facilitate a screening process under water-limiting Mediterranean conditions in breeding programs. The second objective was to identify stress-responsive miRNA and their targets in durum wheat under pre-anthesis water deficit. For this objective [Chapters 4 (Liu *et al.* 2015b) *PLoS One* and 5 (Liu *et al.* 2016b) *Functional & Integrative Genomics*], four closely-related Australian durum varieties with different levels of water-deficit stress sensitivity were used to characterise the durum wheat microRNA transcriptome. Illumina sequencing of 96 small RNA libraries and subsequent analysis identified differentially expressed durum miRNAs in response to water-deficit stress in different tissue types and genotypes. Putative target genes of durum miRNAs were identified *in silico* to predict their possible roles in plant development and stress responses. qPCR examination of durum miRNAs and target genes of interest revealed their complex interactions under water-deficit stress, subject to tissue type and genotype. A 5′ RLM-RACE approach further validated functional genes genuinely cleaved by stress responsive miRNAs in durum wheat. The final objective [Chapter 6 (Liu *et al.* Submitted 2016) *Functional Plant Biology*] was to characterise the temporal pattern of the miR160/Auxin Response Factors (ARFs) regulatory module under water-deficit stress from booting to flowering, and the possible links to physiological traits, morphological traits and yield performance in stress tolerant and sensitive genotypes.

In concluding, this study has significantly enhanced our knowledge surrounding durum wheat miRNAs and their response to abiotic stress constraints such as water deficit. For the first time, this study has characterised the durum wheat miRNA transcriptome and their targets in different tissues of Australian durum genotypes under pre-anthesis water-deficit stress. The collection of papers presented as part of this dissertation provides valuable information

contributing to our understanding of the microRNA-guided regulatory mechanisms underlying stress adaptation in durum wheat.

Chapter 2

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Haipei Liu		
Contribution to the Paper	Drafted the manuscript.		
Overall percentage (%)	60%		
Certification:	This paper is a featured original review article produced during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	22/08/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Amanda Able		
Contribution to the Paper	Drafted the manuscript.		
Signature		Date	22/08/16

Name of Co-Author	Jason Able		
Contribution to the Paper	Drafted the manuscript and acted as the corresponding author.		
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Trends in Plant Science

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Feature Review

SMARTER De-Stressed Cereal Breeding

Haipei Liu,[1] Amanda J. Able,[1] and Jason A. Able[1,*]

In cereal breeding programs, improved yield potential and stability are ultimate goals when developing new varieties. To facilitate achieving these goals, reproductive success under stressful growing conditions is of the highest priority. In recent times, small RNA (sRNA)-mediated pathways have been associated with the regulation of genes involved in stress adaptation and reproduction in both model plants and several cereals. Reproductive and physiological traits such as flowering time, reproductive branching, and root architecture can be manipulated by sRNA regulatory modules. We review sRNA-mediated pathways that could be exploited to expand crop diversity with adaptive traits and, in particular, the development of high-yielding stress-tolerant cereals: SMARTER cereal breeding through 'Small RNA-Mediated Adaptation of Reproductive Targets in Epigenetic Regulation'.

Epigenetic Adaptation to Stress: Beyond the Genes

Abiotic stresses including drought, salinity, and nutrient deficiency threaten plant growth and development, dramatically reducing crop production and quality. Climate change will also impact on the yield potential of key cereal crops such as wheat (*Triticum* spp.), maize (*Zea mays*), rice (*Oryza sativa*), and barley (*Hordeum vulgare*) [1]. Numerous signal transduction pathways prompt adaptive responses at all levels (morphological, physiological, molecular) to help the plant to survive and achieve reproductive success in hostile environments. Reprogramming of gene expression via mechanisms such as epigenetic modification may allow the production or repression of proteins to enable stress adaptation.

Epigenetic modification refers to heritable and transient changes in gene activity and function associated with biochemical modifications of chromatin and **RNA interference** (RNAi) (see Glossary) but does not entail any changes in nucleotide sequence [2–5]. The web of epigenetic regulatory pathways and the interactions therein partially rely on **small RNAs** (sRNAs) to precisely reprogram the expression of stress- or development-associated genes through **transcriptional gene silencing** (TGS) and **post-transcriptional gene silencing** (PTGS) [6–8]. Although other regulatory components (such as long non-coding RNAs and histone modifiers) [9–11] also cause epigenetic modifications associated with stress responses, they are not the focus of this review. Compared with other mechanisms, sRNAs can rapidly respond to different environmental conditions, and act as mobile signal molecules to modulate gene expression during plant development [12–14]. Differential expression of certain sRNAs in response to abiotic stress contributes to the dynamic spatiotemporal patterns of downstream target gene expression and is related to adaptive physiological and/or reproductive traits including altered reproductive timing and alleviation of cellular damage induced by stress in reproductive organs [15–19]. sRNA-mediated regulation may also provide tolerance to recurring abiotic stress through heritable stress memory [20]. Furthermore, responses of the key components in the RNAi mechanism, such as which sRNA families are expressed, appear to be genotype-dependent, thus potentially explaining genotypic differences in their physiological and

Trends

Transcriptome reprogramming and translational regulation involved in plant stress adaptation largely depend on sRNA regulatory pathways, such as transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS).

Crosstalk between sRNA-mediated pathways involved in stress signalling and reproduction has been extended from model plant species to cereal crops.

Desirable reproductive traits such as enhanced panicle branching and more efficient grain filling, and other traits including optimal root architecture in cereals, can be manipulated using RNA interference (RNAi) to maintain/improve yield under challenging conditions.

Newly developed RNAi technologies, such as artificial sRNAs and target mimicry of multifunctional sRNAs, provide new opportunities for stress tolerance improvement in cereals and the intelligent design of high-yielding varieties in molecular breeding.

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morphological stress responses [15,21–24]. Therefore, elucidation of sRNA-mediated epigenetic pathways could be exploited to expand crop phenotypic diversity with favourable physiological and reproductive traits. In this review we discuss the contribution of sRNA regulatory mechanisms to stress adaptation and reproduction in plants, highlighting recent related progress in cereal crops, and evaluate the potential of applying RNAi technologies to developing high-yielding elite cereal varieties.

sRNAs: The Epigenetic Commander Under Stress

Plant small RNAs, mainly **microRNAs** (miRNAs) and **small interfering RNAs** (siRNAs), function as negative regulators in distinct but overlapping epigenetic silencing pathways. The biogenesis of plant miRNAs and siRNAs is relatively well understood, as reviewed recently in [25]. Generally, mature single-stranded miRNAs are processed from precursor miRNAs that originate from hairpin primary-miRNAs, which are transcribed from *MIR* genes. miRNAs can also be produced from intronic or exonic regions of protein-coding genes and transposons [26,27]. siRNAs are derived from long double-stranded (ds) RNA precursors, which originate from DNA repeats, transposons, non-coding loci, and protein-coding genes (exonic and intronic regions) [25,28]. Mature miRNAs and some siRNAs, such as **trans-acting siRNA** (ta-siRNAs), are loaded into the **RNA-induced silencing complex** (RISC) in association with **Argonaute** (AGO) proteins [25]. When bound, RISCs cause sequence-specific cleavage of the complementary target mRNAs and/or translational inhibition, resulting in PTGS [29,30]. Stress-induced, untranslated region (UTR)-derived siRNAs (sutr-siRNAs) could also be functional in the PTGS mechanism through regulation of alternative precursor mRNA (pre-mRNA) splicing [31]. However, siRNAs and, in some cases, miRNAs can reversibly modify chromatin via DNA methylation or histone modification [8] affecting accessibility of chromatin, thus determining whether a particular locus is transcriptionally silent or active [7,32]. Under unfavourable conditions, sRNAs can rapidly respond to different environmental cues and reprogram the expression of downstream genes that provide stress adaptation and heritable stress memory [20]. Sitting at the crossroads of TGS and PTGS pathways, sRNAs are therefore crucial regulators in plant acclimatisation to abiotic stresses (Figure 1).

sRNAs in TGS: Stress-Adaptive Chromatin

In response to environmental and developmental cues, sRNAs help to shape the genotype into the phenotype via stress-responsive regulation of TGS mechanisms such as histone modification and DNA methylation [7,25,33]. sRNAs coordinate histone modification by recruiting enzymes that catalyse the methylation and deacetylation of specific lysine or arginine residues in histones, causing them to be more closely associated to chromatin. Thus the binding of transcription factors to template DNA is limited, leading to suppression of transcription [34,35]. Gene transcription is regulated in this manner in many stress-related processes [35] and, particularly for ABA signalling, cold adaptation, drought adaptation, and the FLC flowering pathway [36–39].

DNA methylation inhibits the transcription of protein-coding genes and transposon movement, which could affect the transcription of neighbouring genes [5,40]. During **RNA-directed DNA methylation** (RdDM), dsRNAs are processed to 21–24 nt siRNAs, which recruit DNA methyltransferases and guide *de novo* methylation by sequence complementarity [25,33]. RdDM machinery has been reported to regulate developmental processes including flowering, ovule development, and male fertility, contributing to reproductive success [33,41,42] and stress-responses to drought and salinity in plants [43,44]. Furthermore, siRNAs appear to contribute to stress tolerance through directing RdDM and modulating DNA methylation in a genotype-dependent manner in rice [43]. Given that the DNA methylation state appears to be heritable [32], the manipulation of siRNAs therefore has potential for breeding stress tolerance.

Glossary

ABCE model: a floral development model. Activity of A genes alone, such as *APETALA2* (*AP2*), leads to sepal formation. Joint activity of A and B genes, such as *AP3* and *PISTILLATA* (*PI*), leads to petal development. Joint activity of B genes with C genes, such as *AGAMOUS* (*AG*), leads to stamen formation but C gene activity alone allows carpel formation. The E genes, or *SEPALLATA* (*SEP*) family, contribute to formation of all floral organs while A and C genes are antagonistic to each other.

Argonaute (AGO): essential catalytic components of the RNA-induced silencing complex (RISC) that bind to different classes of sRNAs and coordinate downstream gene-silencing events.

Artificial microRNA (amiRNAs): artificial sRNAs (21 nt) made using modified backbones of endogenous precursor miRNAs.

CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated nuclease 9): the prokaryotic immune system modified for genome editing. CRISPR are short palindromic repeats of DNA sequences acquired from previous exposure to bacterial, virus, or plasmid invasion. Cas9 is a DNA endonuclease associated with CRISPR that edits the genome with the help of guide RNA which contains user-defined targeting sequences. CRISPR/Cas9-based miRNA knockdown has the exclusive benefit of single-nucleotide precision to differentiate miRNA isoforms in the same family.

MicroRNAs (miRNAs): non-coding, single-stranded RNAs (20–25 nt) transcribed from *MIR* genes. miRNAs induce mRNA cleavage and translational inhibition in a sequence-specific manner.

Post-transcriptional gene silencing (PTGS): repression of gene activity at the post-transcriptional level.

RNA-directed DNA methylation (RdDM): the major epigenetic process involved in biogenesis of small interfering RNA (siRNAs) and DNA methylation. During RdDM, siRNAs are processed from long double-stranded (ds) RNAs.

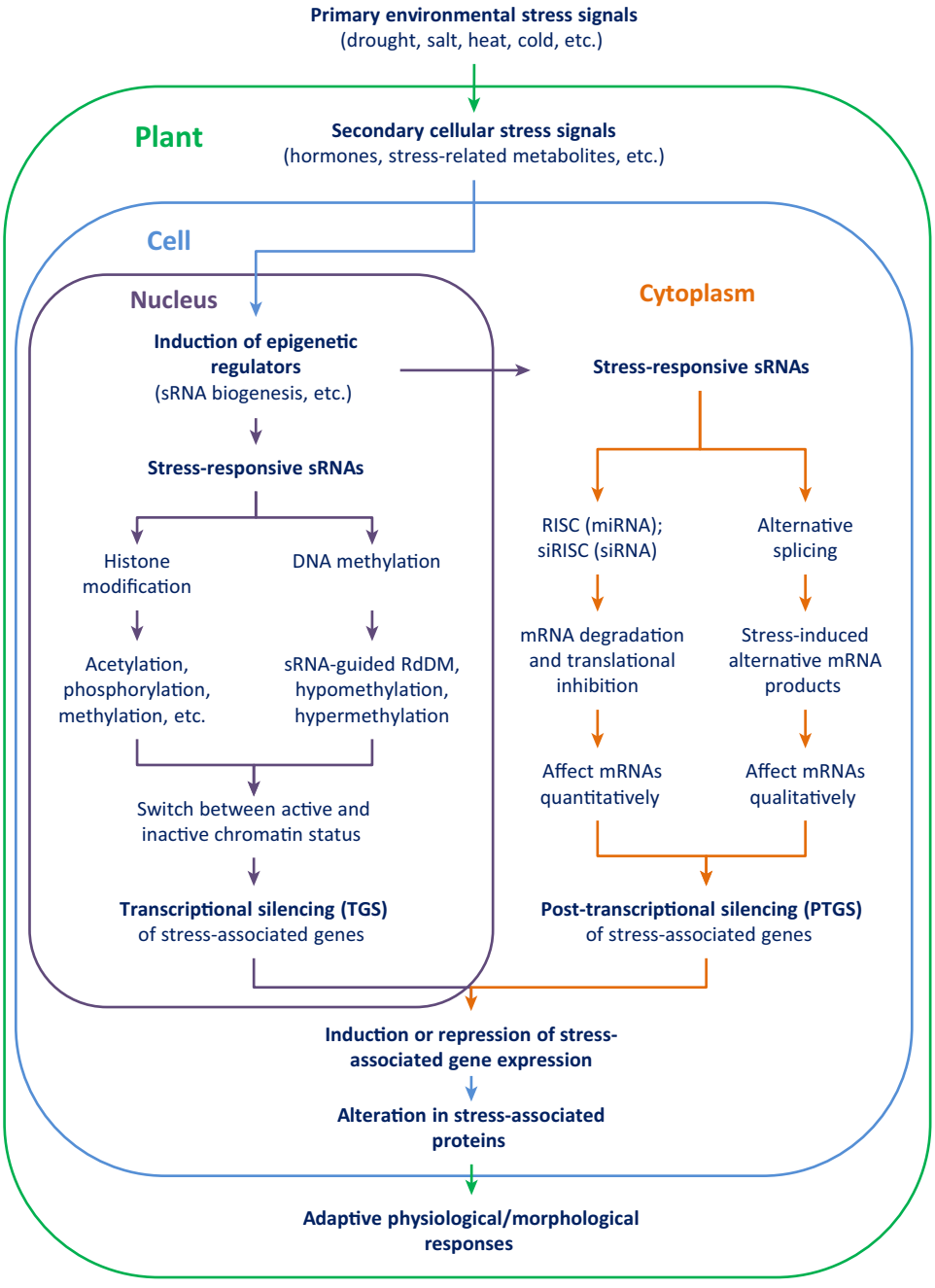


Figure 1. A Systematic View of Small RNA (sRNA)-Mediated Epigenetic Changes Contributing to Gene Expression Reprogramming in Response to Abiotic Stresses. In unfavourable environmental conditions, stress signals are perceived and transduced to plant cells. A network of epigenetic regulation pathways is triggered by cellular stress signals to reprogram gene expression. Stress-responsive sRNAs are induced or repressed in response to various abiotic stresses. Some sRNA families, mainly small interfering RNAs (siRNAs), regulate DNA methylation and histone modification activities which affect chromatin status, leading to transcriptional gene silencing (TGS). MicroRNAs, sometimes siRNAs, are transported into the cytoplasm to guide RISC (RNA-induced silencing complex), leading to post-transcriptional gene silencing (PTGS). Gene expression of functional proteins is downregulated or upregulated through the switching on/off of the TGS and PTGS mechanism under the control of stress-responsive sRNAs. Reprogrammed gene expression leads to downstream physiological or morphological changes in plants contributing to stress adaptation.

RNA-induced silencing complex (RISC): the multi-protein heterogeneous complex that incorporates Argonaute proteins and one guiding strand of a siRNA or miRNA. The guide RNA strand functions as the template in RISC for binding to mRNAs based on sequence complementarity during PTGS.

RNA interference (RNAi): a natural gene-silencing mechanism in which gene expression is repressed by sRNAs through mRNA degradation or inhibition of translation.

Short tandem TM (STTM): STTM is similar to target mimicry but only two miRNA binding sites are linked with a short spacer to deplete and degrade miRNAs in the STTM system.

Small interfering RNAs (siRNAs): sRNA molecules (21–24 nt) processed from long dsRNAs that are mainly generated from the transcription of DNA repeats and transposable elements.

Small RNAs (sRNAs): a large family of small regulatory non-coding RNA molecules (20–50 nt). In plants, sRNAs are integral components of development patterning, maintenance of genome integrity, and plant responses to abiotic and biotic stresses.

Synthetic trans-acting siRNAs (syn-tasiRNAs): siRNAs (21 nt) artificially made by using the modified backbone of an endogenous trans-acting siRNA (tasiRNA) precursor such as TAS1 or TAS3.

Transcription activator-like effector nucleases (TALENs): restriction enzymes containing a TAL effector domain and a specific DNA-binding domain. DNA-binding domain structure can be engineered to bind specifically to target sequences. TALEN-based miRNA knockdown has the advantage of being able to mutate multiple bases.

Target mimicry (TM): a mechanism whereby endogenous non-coding RNAs mimic miRNA-targeted mRNAs and sequester mature miRNAs, relieving their *bona fide* targets from the RNAi machinery.

Transacting siRNA (ta-siRNAs): plant-specific secondary siRNAs produced from transcripts of *TAS* genes with the help of specific miRNAs.

Transcriptional gene silencing (TGS): suppression of gene

transcription through modification of chromatin.

Virus-based miRNA silencing (VbMS): the silencing of endogenous miRNAs using plant viral vectors such as barley stripe mosaic virus to drive TM.

sRNAs in PTGS: Kill the Messenger under Stress

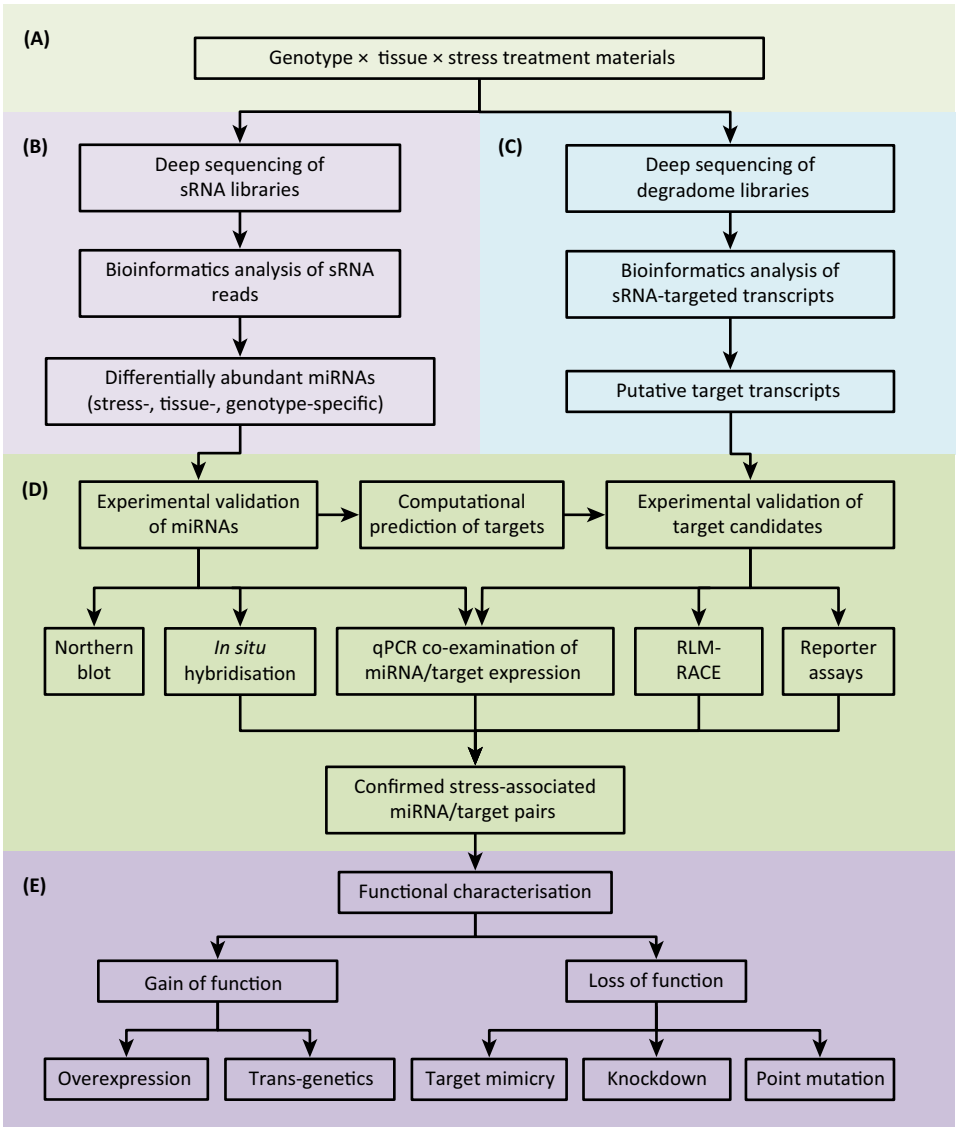
The role of sRNAs (especially sutr-siRNAs and miRNAs) in PTGS during plant stress responses and development has received significant attention. sRNAs act as negative regulators at the post-transcriptional level by affecting the mRNA population both qualitatively and quantitatively via alternative splicing or mRNA degradation, and by preventing protein translation [25].

Sutr-siRNAs appear specific to stress responses and target the genomic intron regions to affect alternative splicing (AS) [31]. The AS mechanism enables the production of multiple mature mRNA isoforms from the same pre-mRNAs but is coupled with the nonsense-mediated decay (NMD) pathway to ensure that nonsense mRNAs generated by AS are degraded. In brachypodium *(Brachypodium distachyon*), a model cereal, sutr-siRNAs were produced from the 3′-UTRs of stress-responsive coding genes under heat, cold, and salt stresses [31]. Sutr-siRNAs target specific complementary *cis*-elements, providing additional splice sites rather than the major annotated splice sites in the target introns, and this ultimately leads to the production of shorter alternative transcripts. These short transcripts possess a stop codon downstream of the sutr-siRNA-targeted splice sites, making them substrates to NMD under stress [31]. Sutr-siRNAs could therefore act as a regulatory switch between non-functional and functional transcripts according to different environmental signals. However, further experimental validation will be necessary to characterise the base-pairing interactions between sutr-siRNAs and their target introns during abiotic stress.

Under environmental stress, plants need to coordinate the balance between developmental patterning and stress defence activation because of the limitation of resources. The abundance of sRNAs, especially miRNAs, regulates gene expression in a highly explicit sequence-specific manner by either causing mRNA degradation or by inhibiting mRNA translation owing to the presence of the RISC that prevents the formation of the ribosomal machinery [45,46]. Development of high-throughput sequencing technology, enhanced bioinformatics tools, and the gradual completion of plant whole-genome sequences has enabled genome-wide analysis of the sRNA transcriptome and its target transcriptome in various plant species [47,48] (Figure 2). The target repertoire of the miRNA-mediated RNAi mechanism includes protein-coding genes involved in a broad range of biological processes {e.g. phytohormone biosynthesis, protein and nucleic acid binding, carbohydrate metabolic processes, protein transport, and ROS (reactive oxygen species) scavenging} [8,49,50]. Several recent reviews have highlighted the specific regulatory roles of different miRNA families in plant defence against environmental stresses [8,49,50]. Some stress-responsive miRNA/target modules also exhibit tissue-specific patterns for their specific roles, including regulating photosynthetic activity and stomatal development in the leaves, and also modulating lateral root initiation and water/nutrient uptake in the roots [15,51,52]. In addition, some miRNA/target modules exhibit opposite regulatory patterns between stress-tolerant and -sensitive varieties or, in some cases, are only active in the stress-tolerant genotypes [15,21–24]. The genotype-dependent nature of these miRNA/target modules and their ability to control stress signal recognition, hormone signal transduction, and downstream stress-inducible regulatory elements leading to physiologically or morphologically adaptive changes makes them promising candidates for crop improvement. Recent assessment of stress-responsive sRNA/target modules in cereal crops has provided valuable information to fully understand the molecular mechanism underlying stress tolerance (Table 1).

sRNA Control of Reproduction: Flourishing Under Stress

Epigenetic regulation coordinated by sRNAs appears to be involved in almost all reproductive processes including phase transition, flowering initiation, inflorescence branching, floral organ development, gametophyte development, and seed/fruit setting [19,21,53–58] (Figure 3, Key Figure). The manipulation of specific sRNA-mediated modules to alter floral initiation, development,



Trends in Plant Science

Figure 2. In Search of Functional Small RNAs (sRNAs) and Their Targets in Plants. (A) Plant materials with different combinations of genotypes, tissue types, developmental stages, and stress-treatments provide multiple options to compare and analyse sRNAs and their targets. (B) High-throughput sequencing of sRNA libraries produced from these materials enable the genome-wide identification of genotype-, tissue-, development-, and stress-dependent functional sRNAs. (C) Degradome sequencing of the 5'-end uncapped RNA fragments is an efficient approach for profiling sRNA-cleaved targets on a large scale. Both sequencing approaches must be integrated with powerful bioinformatics tools to decipher sequencing data, identify valid sRNA/target reads, and characterise the sRNA/target transcriptome profiles. (D) The bioinformatic predictions require experimental validation of sRNA/target candidates to confirm their interactions and (E) characterise their functional relevance. For example, co-examination of sRNA/target pairs using qPCR helps to validate the suppression of mRNAs as a result of changes in sRNA abundance. RLM (RNA ligase mediated)-5' RACE (rapid amplification of cDNA ends) confirms the truncated site in mRNAs resulting from the post-transcriptional gene silencing (PTGS) cleavage guided by sRNAs. Gain-of-function and loss-of-function studies further characterise the roles and relevance of sRNA and their targets in response to stress.

Table 1. Stress-Responsive miRNAs and Their Functional Targets in Cereal Crops

miRNA[a]	The Response of miRNA to Abiotic Stresses[b,c]				Target of miRNA	Pathways Involved	Refs
	Drought	*Salinity*	*Heat*	*Cold*			
miR156	Osa↓, Tae↑, Ttu↑↓, Zma↑	Tae↑↓, Zma↓	Osa↓, Tae↑	Osa↑, Tae↑	Squamosa promoter binding protein-like (SPL) transcription factors	Gibberellin signalling; flavonoid biosynthesis; anthocyanin metabolism	[15,16,18,21,80, 90,137–141]
miR159	Osa↑↓, Tae↑↓, Ttu↓, Zma↑	Hvu↑↓, Osa↓, Tae↓	Osa↓, Tae↓	Tae↑	MYB family transcription factors	Gibberellin signalling	[16,18,21,22,86, 90,137,139, 141–143]
miR160	Osa↑, Tae↑↓, Ttu↑↓	Osa↓, Tae↑↓	Hvu↑, Osa↑, Tae↓	Osa↑, Tae↑	Auxin response factors	Auxin signalling	[15,16,21,90,137, 139–141,144]
miR162	Ttu↑↓	—	Osa↑	—	DICER LIKE 1	Small RNA biogenesis	[22,90,145]
miR164	Osa↑, Tae↑↓, Ttu↑↓	Hvu↑↓, Osa↓, Tae↑↓	Osa↓	Tae↓	NAC domain transcription factors	Hormone signalling	[15,21,22,90,137, 139,141,143,146]
miR166	Osa↓, Tae↑↓, Ttu↑↓, Zma↓	Osa↓, Zma↑	Hvu↑, Osa↑	Osa↓	Homeodomain–leucine-zipper (HD-Zip) transcription factors	Jasmonic acid signalling; ethylene pathways	[15,16,18,21,90, 137,138,144]
miR167	Osa↓, Tae↑, Ttu↑↓, Zma↑	Hvu↑↓, Osa↓, Tae↑, Zma↓	Hvu↑, Osa↓, Tae↑	Osa↑, Tae↓	Auxin response factors	Auxin signalling	[15,16,18,21,90, 137–141,143,144]
miR168	Hvu↓, Osa↓, Tae↑, Ttu↑↓, Zma↓	Hvu↑↓, Tae↑, Zma↑		Tae↑	Argonaute 1	RISC loading; ABA signalling	[15,18,21,137,138, 141–143,147]
miR169	Hvu↓, Osa↑, Tae↑↓, Ttu↓	Hvu↑↓, Tae↑↓, Zma↑	Osa↓	Tae↓	NF-YA transcription factors	ABA biosynthesis; ABA signalling	[21,22,90,138,139, 141–143,147]
miR171	Hvu↓, Osa↑↓, Tae↓, Ttu↑↓	Hvu↑↓, Tae↑	Tae↓, Osa↑↓	Tae↑	SCARECROW-like (SCL) transcription factors	Gibberellin signalling	[15,21,22,90, 140–143,147]
miR172	Hvu↓, Osa↓, Tae↑↓	Hvu↑↓, Tae↑	—	—	APETALA2 (AP2) and AP2-like transcription factors	ABA biosynthesis and signalling; meristem establishment	[21,139,142, 143,147]
miR319	Osa↑↓, Tae↑↓, Ttu↓, Zma↑	Hvu↑↓, Osa↓, Tae↑	Tae↓	Tae↑	TCP family transcription factors	Jasmonate biosynthesis and senescence	[16,18,21,22,137, 139–143]
miR393	Hvu↓, Osa↑, Tae↑↓, Ttu↓	Hvu↑↓, Osa↑, Tae↑	Osa↑	Osa↑, Tae↑	TIR1 (transport inhibitor response 1) proteins, AFB (auxin signalling F-box) proteins	Auxin signalling; auxin homeostasis	[16,21,22,90,137, 141,143,147]
miR394	Osa↑, Ttu↓↑	Osa↓	—	Osa↑	F-box domain-containing proteins	ABA signalling	[15,16,137]
miR395	Osa↑, Tae↓, Ttu↑↓, Zma↓	Hvu↓, Tae↑, Zma↑	Tae↑	Tae↓	ATP sulfurylase genes, SULTR2;1 (sulfate transporter 2;1) protein	Sulfate transport and assimilation	[15,18,21,138–143]
miR396	Hvu↓, Osa↑↓, Ttu↑↓, Tae↑↓, Zma↓	Hvu↑↓, Osa↓, Tae↑↓, Zma↓	Osa↑	Osa↑, Tae↑	GRF (growth-regulating factor) proteins, bHLH74 (basic helix-loop-helix transcription factor 74)	Cell proliferation	[15,16,18,21,22,90, 137–139,141–143, 147–149]
miR397	Hvu↓, Osa↑↓, Ttu↑	Tae↑	Osa↑	Tae↑	Laccase (LAC) genes	Brassinosteroid sensitivity; cell wall biosynthesis	[15,24,90,141,147]
miR398	Osa↑↓, Ttu↑↓, Zma↑	Tae↓, Zma↑	Osa↑, Tae↑	Tae↓	CSDs (Cu/Zn superoxide dismutases)	Reactive oxygen species (ROS) scavenging	[15,18,24,90,138, 140,141]

Table 1. (continued)

miRNA[a]	The Response of miRNA to Abiotic Stresses[b,c]				Target of miRNA	Pathways Involved	Refs
	Drought	*Salinity*	*Heat*	*Cold*			
miR399	Osa↑, Ttu↑↓, Zma↓	Tae↑, Zma↓	Osa↓	Tae↑	Ubiquitin-conjugating (E2) enzymes	Cellular phosphate homeostasis; phosphate remobilisation	[15,18,90,137,141,148]
miR408	Osa↑↓, Ttu↑↓	Hvu↓, Tae↑	Tae↑	Tae↑	Plastocyanin-like (basic blue) proteins, TOC1	Copper homeostasis; cell-to-cell signalling	[15,23,140,141,143]
miR444	Hvu↓, Osa↑, Tae↑↓, Ttu↓	Hvu↑↓, Tae↓	—	Tae↑	MADS-box transcription factors	Cellular nitrate signalling	[15,21,137,139,141, 143,147]
miR528	Osa↑↓, Ttu↑↓, Zma↓	Osa↓, Zma↑	—	Osa↓	AAO (ascorbic acid oxidase), laccase precursor proteins, CSDs	Oxidation/reduction processes	[15,16,18,24,137,148]
miR529	Osa↑↓	Osa↑, Tae↓	Osa↓	Osa↑	SPL transcription factors	Gibberellin signalling	[16,90,137,139,142]
miR827	Hvu↓, Osa↓, Tae↑↓, Ttu↑↓, Zma↑	Hvu↑↓, Zma↓	—	—	SPX-MSF genes	Cellular phosphate homeostasis	[15,18,21,137,143, 147,148]
miR1029	Tae↑	—	Tae↑	Tae↓	DRE-binding factors, AP2-like transcription factors	Gibberellin biosynthesis; ABA signalling	[150]
miR1030	Hvu↓, Osa↓	Tae↑	—	—	—	—	[141,142,147]
miR5048	Hvu↓, Ttu↑↓	Hvu↑↓	—	—	Cysteine-rich receptor-like protein kinases	—	[15,143,147]
miR5049	Hvu↑↓, Ttu↑↓	Tae↓	—	—	Ubiquitin-conjugating (E2) enzymes	—	[15,139,147]
miR5064	Hvu↓, Ttu↑↓	—	—	—	—	—	[22,147]
miR5072	Hvu↓	Hvu↑↓	—	—	Anthocyanidin reductase	—	[143,147]
miR6300	Hvu↑, Ttu↑↓	—	—	—	—	—	[15,147]

[a]miRNAs that are also discussed in this review for their role in the regulation of plant development are in italic font.
[b]Abbreviations: Hvu, *Hordeum vulgare*; Osa, *Oryza sativa*; Tae, *Triticum aestivum*; Ttu, *Triticum turgidum*; Zma, *Zea mays*.
[c]Symbols: ↑, upregulated; ↓, downregulated; —, not determined; ↑↓, opposite regulatory patterns observed in different studies; ↑↓, opposite regulatory patterns observed between stress-tolerant and stress-sensitive genotypes in the same study.

and grain fill therefore holds great potential as a tool to facilitate reproductive success and yield improvement under different environmental conditions (Figure 3).

Flowering Time and Floral Development

Regulatory networks controlling the vegetative to reproductive phase transition are highly complex and regulated strongly by environmental cues [59,60]. These networks have been well studied in arabidopsis (*Arabidopsis thaliana*), as reviewed recently [59], to reveal the importance of a key set of genes that integrate pathways to initiate flowering: *SUPPRESSOR OF CONSTANS 1* (*SOC1*), *FLOWERING LOCUS T* (*FT*), and *AGAMOUS-LIKE 24* (*AGL24*). These genes then switch on a number of floral meristem (FM) identity genes including *APETALA1* (*AP1*), *LEAFY* (*LFY*), and *FRUITFULL* (*FUL*) leading to FM development. Both sets of genes have been shown to be regulated by various genes associated with environmental cues, including *TIMING OF CAB EXPRESSION 1* (*TOC1)* (circadian clock), *FLOWERING LOCUS* C (*FLC)* (vernalisation), *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes, *TARGET OF EAT1* (*TOE1*) (age), and *DELLA* (gibberellins). The FM then gives rise to various floral organ primordia, a process directed by floral organ identity genes often represented in the **ABCE model** [60,61].

Key Figure

Key MicroRNA (miRNA)-Mediated Regulatory Modules Involved in Plant Development and Reproduction

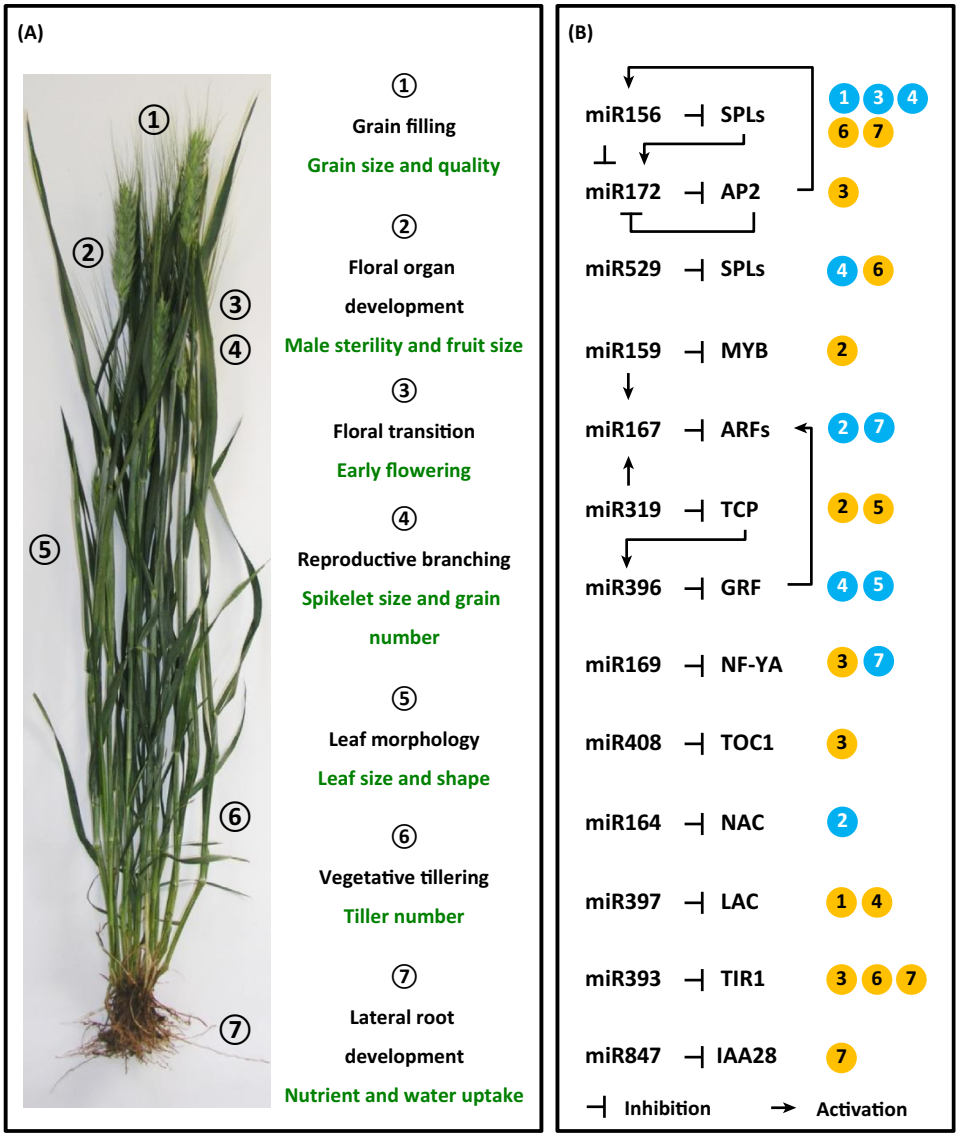


Figure 3. For a Figure360 author presentation of Figure 3, see the figure online at http://dx.doi.org/10.1016/j.tplants.2016.07.006#mmc1.

(A) Plant development stages and potential breeding targets. Seven development-associated events are indicated and numbered. Green text refers to favourable physiological and reproductive traits in breeding that could potentially be engineered during plant development. (B) Key miRNA/target regulatory modules discussed in the review that could be used as a tool to manipulate plant development and reproduction. Numbered plant developmental stages highlighted in blue indicate the positive regulation of development by miRNA-targeted genes. Numbered plant development stages highlighted in orange indicate the negative inhibition of development by miRNA-targeted genes.

Knowledge of the gene network responsible for floral timing and development is equally important in cereal breeding where flowering time affects pollination, seed quality, yield, harvest time, and stress avoidance. Although many of the regulatory network components, primarily transcription factors, described in arabidopsis have also been identified in cereals [59], no functional *FLC* orthologues have been validated in cereals thus far. In arabidopsis, vernalisation or the acceleration of flowering via cold temperatures requires epigenetic silencing of the floral repressor gene *FLC* [62,63] by sRNAs, histone modifiers, and long non-coding RNAs [11,62–64]. Major crops such as winter wheat and barley respond to vernalisation by increasing the expression of *VRN1* (similar to *AP1/FUL*), which usually represses *VRN2*. Because *VRN2* (a zinc-finger CCT domain-containing gene) usually represses the *FT* orthologue, *VRN3*, flowering is initiated. Even though a TamiR1123 (previously named miR507) was identified to originate from miniature inverted-repeat transposable elements in the *VRN*-A1a promoter, the role of cereal miRNAs in these networks remains unknown [59,65] (see Outstanding Questions).

The miR156/157 and miR172 families are probably the best-characterised flowering time regulators because their functions are highly conserved across dicots and monocots. These two miRNA families exhibit temporally opposite expression patterns and have inverse functions in regulating floral time [66]. miR156 is highly expressed in the vegetative stage and its abundance gradually declines as the plant ages, whereas miR172 accumulates over time in leaves and floral organs from the vegetative to reproductive stage [67]. The main targets of the miR156/157 family are *SPL* transcription factors and, in arabidopsis, rice, maize, and brachypodium, they appear to regulate vegetative/reproductive phase transition, inflorescence branching, and axillary meristem boundary establishment [66,68–70]. Indeed, overexpression or upregulation of miR156 (and therefore decreased *SPL* expression) led to delayed flowering and a prolonged vegetative phase in several species (reviewed in [53]). miR172 has been shown to target the 'A' gene *AP2 (APETALA2)* [71], and *AP2*-like genes including *TOE1*, *TOE2*, *TOE3*, *SMZ*, and *SNZ* [72–74]. In arabidopsis, overexpressing miR172 led to increased cleavage of *TOE1, TOE2*, and *AP2* [74], and caused early flowering [73]. Overexpression of rice miR172 also significantly reduced flowering time through its repression of two *AP2* genes, *SUPERNUMERARY BRACT* (*SNB*) and *INDETERMINATE SPIKELET 1* (*IDS1*) [71]. Interestingly, the AP2 transcription factor negatively regulates miR172 expression and positively regulates miR156 expression, forming a well-coordinated feedback loop [75]. Moreover, miR156 indirectly regulates miR172 abundance because some *SPL* genes, such as *SPL9* and *SPL10*, induce transcription of miR172 [66]. The temporal pattern of miR172 increasing with age could therefore be the direct consequence of reducing miR156 and increasing *SPL*s [66]. Nutrient availability is also involved in this developmental timing feedback loop through sugar-mediated repression of miR156 [76], and miR156-mediated responses to phosphate starvation [77]. Furthermore, environmental factors such as drought and heat affect flowering time through increased biogenesis of miR172 induced by GIGANTEA and FCA proteins, respectively [78,79], as well as miR156-regulated stress response and memory [20,80]. The regulatory circuit between miR156/157, miR172, and their targets therefore appears crucial for floral transition. Furthermore, because *SPL*s have diverse functions across plant development, miR156 appears to be important in other aspects including inflorescence development. In rice, *SPL14* is encoded by the quantitative trait locus, *IPA1* (*IDEAL PLANT ARCHITECTURE 1*) [81,82]. Interruption of miR156-directed binding of *IPA1* via a point mutation in *OsSPL14* caused a marked accumulation of *IPA1*, leading to denser panicles with more primary and secondary branching, and therefore more grain [81]. IPA1 affects inflorescence development by activating transcription of *TB1* (*TEOSINTE BRANCHED 1*, a negative regulator of tiller bud outgrowth) and *DEP1* (*DENSE AND ERECT PANICLE 1*, a positive regulator of panicle architecture and panicle length) [83]. In rice, another miRNA (miR529) also targets the *SPL* family, affecting panicle size. Rice plants overexpressing miR156 or miR529 exhibited significantly increased tillers and smaller panicles but with less reduction caused by miR529 [57]. Interestingly, miR529 appears to be specific to

monocots [84]. However, in arabidopsis, *AtSPL9* and *AtSPL15* retain the target site of miR529 and were still responsive to regulation by osa-miR529. The evolutionary relatedness of miRNA/target modules could therefore be used when considering their transfer between dicots and monocots for floral engineering (see Outstanding Questions).

Another characterised miRNA regulatory circuit in floral development involves miR159, miR319, and miR167 [85]. miR159-regulated MYB transcription factors and miR319-regulated TCP transcription factors have overlapping functions in floral organ development [85,86], and can independently induce the expression of miR167, which in turn represses *AUXIN RESPONSE FACTOR 6* (*ARF6*) and *ARF8*. Both of these participate in auxin signalling, cytokinin activity, and the activation of jasmonic acid biosynthetic enzymes [85]. Impairment of miR159 and miR319 through target mimicry led to defects in sepals, petals, stamen, and anthers, which interestingly resembled the defects caused by the reduced activity of *ARF6/8* when miR167 was enhanced [85]. In addition, overexpressing tae-miR159 in rice resulted in delayed heading time and male sterility [86], probably owing to the role that the target MYBs play in stamen and anther development [85,87]. Furthermore, in the maize *dicer-1 like* mutant, *fuzzy tassel*, downregulation of miR159 and subsequent misregulation of its target mRNA, gibberellin (GA)-induced MYB, led to male sterility [87]. The modulation of the miR159–miR319–miR167 regulatory circuit might therefore be useful when considering the creation of male-sterile lines for F_1 hybrid production in breeding programs.

Because plants will flower earlier in response to stress [88], miRNAs identified as being upregulated during abiotic stress might also control flowering. These include the previously discussed miR156–miR172 regulatory circuit, as well as miR169 and miR408 family members which target key components of the floral regulatory network. The miR169 family targets the universal transcription factor subunit NF-YA (nuclear factor Y subunit A), which binds to the promoter and first intron of the *FLC* gene and induces its transcription [19] while miR408 appears to target the circadian clock gene *TOC1* [54]. In arabidopsis and wheat, most members of the miR169 family are upregulated in response to abiotic stress [19,21]. However, in maize roots and rice panicles, miR169 showed decreased abundance under abiotic stress [89,90]. Therefore, the miR169/NF-YA module may not necessarily be ideal for the control of stress-induced flowering. However, miR408 overexpression in wheat has shown some promise for future application [54], with knockdown of *TOC1* expression leading to an early-heading wheat phenotype [54], and therefore the possibility of avoiding the usual stresses that occur during grain development such as water deficit stress [15] and heat stress [91]. Interestingly, bioinformatics analysis indicated that the miR408 targeting site in *TOC1* also exists in barley, but could not be found in rice, maize, brachypodium, soybean (*Glycine max*), or arabidopsis. Furthermore, in arabidopsis, overexpressing tae-miR408 did not repress *TOC1* [54]. Consequently, the manipulation of this miRNA regulatory module in adjusting heading time may be applicable only in particular cereal species.

The cautionary tale of understanding multiple functions of specific miRNA modules to avoid undesirable side effects continues with miR164 which appears to be crucial for defining morphogenetic floral organ boundaries in developing flowers [55,56,92] through its ability to downregulate various NAC-domain transcription factor families [56,93]. However, miR164-targeted NAC genes also negatively regulate drought resistance in rice and stripe rust resistance in wheat [93,94]. Therefore, enhancement of miR164 expression in these crops could contribute to stress resistance, but might cause undesirable reproductive defects.

The miR396 and miR397 families are also influenced by abiotic stresses (Table 1), but both appear to integrate inflorescence development, auxin biosynthesis, and hormone signalling pathways [58,95]. For example, osa-miR396 targets *GROWTH REGULATING FACTOR 6*

(*OsGRF6*), which functions in auxin biosynthesis and activates auxin response factors and branch/spikelet development-related transcription factors [95]. Increased grain yield occurs in rice plants with knocked-down miR396 because enhanced expression of *OsGRF6* promotes the formation of axillary branches and spikelets [95]. Likewise, increased grain yield occurs in rice plants overexpressing osa-miR397, but this is due to enhanced panicle branching and larger grain size. In the case of osa-miR397, it represses *LACCASE-LIKE PROTEIN* (LAC) which is involved in brassinosteroid sensitivity and cell wall biosynthesis [58]. Clearly, miRNAs such as these are therefore not only important in controlling floral development but also in modulating events downstream of fertilisation such as embryo and endosperm development, often referred to as grain filling in cereals.

Grain Filling

sRNA profiling in rice, wheat, barley, and maize has demonstrated that various sRNA families, especially miRNAs, exhibit spatiotemporal patterns of expression during grain development [17,96–99]. These differentially expressed miRNAs and their targets are mostly involved in multiple signalling and biosynthetic pathways such as hormone homeostasis and starch biosynthesis, which could contribute to coordinated nutrient accumulation in the growing endosperm. For example, in rice, a quantitative trait locus *GW8* (synonymous with the miR156-targeted *OsSPL16*) encodes a protein that is a positive regulator of cell proliferation [100]. Increased expression of *OsSPL16* promoted cell division and grain filling, and this led to enlarged endosperm size, grain width, and increased yield in rice. As mentioned earlier, the manipulation of miR397 in rice also enhanced grain filling and generated larger grains, ultimately contributing to a 25% increase of grain yield in field trials [58]. Some miRNA families, including miR156, miR164, miR167, miR397, miR1861, and miR1867, have higher abundance in superior spikelets (earlier flowering, faster grain fill) [96,101]. By contrast, 24 nt siRNAs showed higher abundance in inferior spikelets (later flowering, slower grain fill) [101]. These 24 nt siRNAs were more likely to be involved in the RdDM pathway, or to more effectively compete for 2'-OH methylation to enable stabilisation [102,103], such that miRNAs will degrade more quickly and therefore lead to a lower abundance of miRNAs in the inferior spikelets [101]. Hence, repression of 24 nt siRNAs could contribute to miRNA accumulation, which might enhance the grain filling rate in inferior spikelets and produce better-quality grains.

sRNA Engineering in Crops: Leap-Frogging Through the Field

Achieving high yield in crops not only relies on adaptive reproductive traits under unfavourable environments but also on agronomic traits such as leaf morphology, root architecture, and tiller branching/number. Leaves with increased photosynthetic efficiency contribute greatly to nutrient accumulation and grain setting rate during reproduction, while a well-developed, well-adapted root system spatially deploys lateral roots and primary roots to optimise water and nutrient uptake. Tiller dynamics, including density and spatial distribution, could affect plant gas exchange, canopy temperature, and also light interception. Most importantly, the fertile tiller ratio and the development of grain-bearing tillers can directly determine the final yield in cereal crops. The involvement of sRNAs in these traits provides new options for researchers to engineer crop architecture, leading to improved plant fitness, subsequent reproductive success, and high grain yield (Figure 3).

A regulatory miRNA circuit involving miR319, miR396, and their respective targets – the *TCP4* and *GRF* genes – appears to play a conserved role in leaf development. In arabidopsis, *TCP4* has been shown to repress cell proliferation, causing a negative impact on leaf size as a result of reduced leaf cell number [104]. However, GRF proteins promote cell proliferation in the meristem and developing leaves [105,106]. The accumulation of *TCP4* induces the expression of miR396, leading to downregulation of *GRF*s and subsequent repression of cell proliferation [107], as does overexpressing miR396 family members in arabidopsis and rice [106–108]. The upregulation of

miR319 could therefore repress the expression of miR396 and alleviate its negative impacts on GRF proteins and leaf development. In rice, the overexpression of two miR319 family members led to increased longitudinal leaf veins and wider leaf blades, and also enhanced cold tolerance [109]. Similarly, overexpression of osa-miR319 in creeping bentgrass (*Agrostis stolonifera*) caused formation of thicker and more-expanded leaves with increased leaf wax, which contributed to enhanced salt and drought tolerance [110]. Given their conserved functions across plant species, the miR319/TCP and miR396/GRF modules could serve as evolutionary RNAi targets to modify leaf morphology.

Several sRNA-mediated pathways also regulate root development through their roles in auxin signalling and can impact on nutrient and water uptake. Overexpressing miR393 and the knockdown of its targets (the auxin receptor genes *AUXIN-BINDING F-BOX 2* and *TIR1*) in rice plants produced similar phenotypes, with significantly longer primary roots and reduced crown roots, typical root traits associated with altered auxin signalling [111]. However, overexpression of miR393 increased grain-bearing tillers and early flowering in rice, but led to reduced tolerance to salinity and drought [112]. Arabidopsis plants overexpressing miR156 produced more lateral roots, whereas reducing miR156 abundance led to less lateral roots through regulation of *SPL*s involved in auxin signalling [113]. In rice, overexpression of miR156 also increased tiller number and reduced plant height [57,70], but ectopic expression produced a higher fertile tiller ratio, larger panicles, increased grain setting rate, and significant grain yield improvement through the regulation of *SPL*s as mentioned previously [70,81]. Likewise, miR167 overexpression in soybean to downregulate *ARF6* and *ARF8*, and overexpression of miR847 in arabidopsis to downregulate IAA28 (which normally represses *ARF* expression), increased total lateral root number and increased lateral root length [114,115]. Furthermore, the alleviation of miR169-directed repression of NF-YA increased lateral root initiation in arabidopsis [116]. Because miR169 is downregulated under low nitrogen (N) and phosphorous (P) conditions [117,118], knockdown of miR169 may allow increases in N and P uptake through enhanced lateral root development, ultimately leading to improved grain yield and quality. Indeed, overexpressing NF-YA in wheat significantly increased both N and P uptake [119]. Similarly in rice, the miR166-targeted transcription factor RDD1 promotes the uptake and accumulation of various nutrient ions in the roots [120]. The impairment of miR166/RDD1 binding through nucleotide substitution within the miR166 target recognition site produced constitutive RDD1 expression, which ultimately increased nitrogen responsiveness and grain production in rice [120]. Therefore, several candidate regulatory RNAi/target modules exist for improvement of grain yield and quality through the manipulation of leaf morphology, tillering, and root architecture. However, care must be taken to avoid undesirable effects on other traits.

Significant Potential of sRNA Technologies

As a natural mechanism for genetic reprogramming, sRNA-directed RNAi has emerged as a powerful biotechnological tool for gene silencing studies in functional genomics. The use of various RNAi methods has assisted researchers to modify stress responses and reproductive processes in plants, and also expands the power of RNAi in developing high-yielding superior crop varieties.

Several RNAi approaches such as **artificial microRNAs** (amiRNAs) [121–123], **synthetic ta-siRNAs** (syn-tasiRNAs) [124], and the overexpression of *MIR* genes [54,58,109,112] are powerful tools to activate gene silencing through inducing endogenous or exogenous sRNAs. Conversely, the activity of miRNAs can be sequestered using approaches such as sRNA **target mimicry** (TM) [95,125–127], **short tandem TM** (STTM) [128–130], **virus-based miRNA silencing** (VbMS) [131], and **transcription activator-like effector nuclease** (TALEN)-based or **CRISPR/Cas9**-based knockdown of sRNAs [132,133]. TM-based approaches, amiRNAs, and miRNA overexpression, which can all directly modify mature miRNA abundance, are so far the most promising for manipulating reproduction and stress tolerance in crops (Table 2).

Table 2. Current Progress of RNAi Applications in Crop Improvement

RNAi Method	Advantages	Disadvantages	Examples	Refs
Artificial miRNAs	Very effective in knock-down/knock-out studies Few off-target effects Customised to silence both coding and non-coding genes	Not applicable at the DNA level Needs to be combined with tissue-specific promoters to improve efficiency	Improvement of plant height and panicle exsertion to facilitate hybrid rice production	[121–123]
			Control of root architecture through targeting *ETHYLENE RESPONSE FACTOR* genes (*ERF*s)	
			Resistance to Wheat dwarf virus (WDV) through targeting conservative WDV sequences in barley	
Overexpression of miRNAs	Easy to reveal miRNA function through gain-of-function Does not need artificial sRNA constructs	Not very effective for miRNA family members with functional redundancy Needs to be combined with tissue-specific promoters to improve efficiency	Overexpression of miR319 promoted leaf morphogenesis and improved cold tolerance in rice	[54,58,109,112]
			Overexpression of miR393 improved salt and drought tolerance in rice	
			Overexpression of miR397 promoted panicle branching and increased grain size in rice	
			Overexpression of miR408 promoted early heading in wheat	
Target mimics	Easy to generate for their simple structure Very effective in attracting endogenous miRNAs intended to be knocked down	Do not completely degrade mature RNAs Not very effective on highly abundant miRNAs or miRNA family members with functional redundancy	Target mimic of miR156 increased *OsSPL13* to control grain size in rice	[95,125–127]
			Target mimic of miR396 generated higher root biomass and highly-efficient colonization in *Medicago truncatula*	
			Target mimic of miR396 increased secondary branches and spikelets and improved yield in rice	
			Target mimic of miR5200 regulated photoperiod-mediated flowering time in brachypodium	
Short tandem target mimics (STTM)	Effective degradation of mature RNAs through the small degrading nucleases	Not very effective on miRNAs with low abundance	STTM degradation of miR1848 modulated phytosterol and brassinosteroid biosynthesis during plant development and stress response in rice	[128–130]
			STTM degradation of miR396 generated larger reproductive organs and increased fruit yield in tomato	
			STTM blockage of miRNA858 induced anthocyanin biosynthesis in tomato	

Exogenous amiRNAs function in PTGS similar to endogenous miRNAs, but their sequence complementarity can be custom-made to target almost any gene. For example, in rice, the role of *Ghd7* (*Grain number, plant height, and heading date 7*) in regulating heading date, reproductive development, and stress response was revealed by introducing ami-Ghd7, an amiRNA designed complementary to *Ghd7* [134]. Apart from assessing gene function, modifying sRNA could also directly improve agronomically valuable traits. For example, overexpression of amiRNAs results in 80% reduction in the expression level of its target *BETAINE ALDEHYDE DEHYDROGENASE* 2 (*BADH2*), which led to increased 2-acetyl-1-pyrroline, the major compound generating grain fragrance in rice that brings high market value [135]. However, as mentioned earlier, constitutive expression of amiRNAs or overexpression of endogenous miRNAs may also generate undesirable phenotypes. Utilisation of suitable tissue-specific and stress-inducible promoters could adjust gene activity in a more controlled manner, thus minimising undesirable side effects. For example, while flowering can be delayed by silencing *FT* with ami-FT, if an alcohol-inducible promoter is used, flowering could be induced synchronously upon exogenous application of ethanol [136]. However, the design of successful tissue-specific or stress-inducible promoters is challenging. Given that amiRNA-mediated RNAi is a quantitatively effective approach, future development and careful selection of transgenic promoters is very important to fully unlock the potential of amiRNA in crop improvement.

Concluding Remarks and Future Perspectives

Small RNA-mediated epigenetic regulation is involved in almost all biological and metabolic processes during the plant life cycle. Many of these processes are crucial to the maintenance of plant fitness and reproductive success under stressful environmental conditions. Recently characterised sRNA-regulated modules playing decisive roles in reproductive events such as flowering time, panicle branching, and grain development have emerged as a resourceful genetic reservoir for manipulating these challenging breeding targets. However, the contribution of some sRNA families to stress responses and plant development, as well as their trans-generational inheritance and the stability of acquired sRNA-mediated responses, remains unclear (see Outstanding Questions). Furthermore, most of our understanding of stress-induced epigenetic regulation and its adaptive value has been generated from laboratory studies with arabidopsis and rice. Under these conditions plants are often exposed to acute and controlled levels of one single stress, whereas in the field combinations of different abiotic stresses occur simultaneously. The systematic study of sRNA-mediated regulatory mechanisms, and their function, under field conditions for commercial crop cultivation is therefore necessary. Inheritable epigenetic changes, such as DNA methylation and histone variants, could also be exploited, but trans-generational memory of epigenetic variation induced by sRNAs differs according to the environment [7]. Therefore, the benefits and risks of these stress-induced adaptations must be examined in the progenies under different conditions based on their intended regions of cultivation. Together with the identification and characterisation of suitable sRNA/target modules, crops could be manipulated using the various RNAi-based approaches discussed earlier to modify gene expression associated with stress responses and plant reproduction in a controllable manner. These sRNA-associated approaches, together with the development of suitable constitutive, stress-inducible, and tissue-specific RNAi promoters in crop species, could become a sustainable strategy. Furthermore, once whole-genome sequences are available for all species, the full potential of RNAi should be unlocked. SMARTER breeding, through the utilisation of 'Small RNA-Mediated Adaptation of Reproductive Targets in Epigenetic Regulation', could be one of the most promising solutions to improving agricultural productivity by engineering elite crop varieties with enhanced stress tolerance and increased grain yield.

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Outstanding Questions

During domestication, the selection process focusing on high yield performance has considerably limited the genetic diversity of modern crop cultivars. Has this process caused differences in the sRNA mechanisms between cultivars, landraces, and wild relatives? Given the importance of epigenetic regulation in stress adaptation, what are the best ways to exploit sRNA mechanisms among the diverse gene pool available to modern cereal breeding?

Even though *FLC*-like genes have been identified in wheat and barley, to date there is no functional *FLC* validation in cereals. Despite characterisation of the vernalisation-associated *VRN1*, *VRN2*, and *VRN3* (homologue of *FT*) genes in wheat and barley, the epigenetic regulatory mechanism underlying vernalisation is poorly understood. What roles do cereal sRNAs play in this alternative flowering regulatory mechanism governed by the VRN genes?

How did species-specific miRNA regulatory modules such as miR156/529 and their targets (*SPL*/*SBP*-box genes) evolve differently for dicots and monocots? What role do these miRNA regulatory circuits play in the phenotypic changes and speciation that differentiate dicots and monocots? With answers to these questions, could the natural loss of functionality of crucial sRNA regulators be compensated by RNAi manipulation of their evolutionary orthologues, which would concomitantly provide increased options to alter morphological and reproductive traits across dicot and monocot species?

Given the intricacy of complex cereal genomes, would researchers be able to regulate RNAi activity at a chromosome-specific level? Can the accuracy of RNAi-based approaches be improved when targeting individual homoeologous genes with high sequence conservation?

What is the best way to minimise the undesirable pleiotropic effects in RNAi-engineered crops? What is the best way to modify a single trait when a master regulator sRNA controls multiple changes in plant morphology and development?

How can RNAi-conferred stress tolerance in progenies be efficiently and

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accurately evaluated under field conditions given the complex nature of genotype × environment interactions?

What is the best way to maintain RNAi-modified lines that exhibit reproductive abnormality but have other desirable traits such as stress tolerance? These lines and their progeny may be of value to germplasm collections in breeding programs. Alternatively, they may provide opportunities to develop new breeding technologies related to male sterility and F_1 hybrid development.

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Chapter 2 Addendum

Supplementary materials available online via DOI

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Please click on the above DOI link or scan the QR code to download the following supplementary materials. The format of these files is not suitable for thesis binding.

mmc1 (Multi-media component 1): A Figure360 author presentation of Figure 3

mmc2 (Multi-media component 2): Interactive Questions

Chapter 3

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By signing the Statement of Authorship, each author certifies that:

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Morphological, physiological and yield responses of durum wheat to pre-anthesis water-deficit stress are genotype-dependent

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Abstract. Durum wheat production in southern Australia is limited when water deficit occurs immediately before and during anthesis. This study was conducted to determine the effect of genotypic variation on various yield, morphological and physiological responses to pre-anthesis water-deficit stress by evaluating 20 durum wheat (*Triticum turgidum* L. ssp. *durum*) genotypes over 2 years of glasshouse experiments. Grain number was the major yield component that affected yield under pre-anthesis water-deficit stress. Genotypes with less yield reduction also had less reduction in chlorophyll content, relative water content and leaf water potential, suggesting that durum genotypes tolerant of water-deficit stress maintain a higher photosynthetic rate and leaf water status. Weak to moderate positive correlations of morphological traits, including plant height and fertile tiller number, with grain number and biomass make the evaluation of high-yielding genotypes in rainfed conditions possible. Morphological traits (such as plant height and tiller number) and physiological traits (such as chlorophyll content, relative water content and leaf water potential) could therefore be considered potential indicators for indirect selection of durum wheat with water-deficit stress tolerance under Mediterranean conditions.

Additional keywords: chlorophyll content, leaf water potential, morphological traits, relative water content, yield components.

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Introduction

Durum wheat (*Triticum turgidum* L. ssp. *durum*) is a tetraploid wheat species grown commercially, primarily in the North American Great Plains, Mediterranean Europe, Northern Africa, and Australia (Habash *et al*. 2009). Many of these environments are water limiting; therefore, tolerance to water-deficit stress is an important objective in durum breeding globally. The total effect of water deficiency on grain yield of durum wheat is variable because of the unpredictable density, fluctuating amount and, especially, timing of rainfall relative to the crop growth cycle (Dolferus *et al*. 2011). Post-anthesis water stress has severe effects on the grain-filling process and grain size due to the change in dry matter accumulation when grain number is already established (Yang and Zhang 2006; Ercoli *et al*. 2008). Water-deficit stress at heading has been shown to reduce the number of grains per spike by increasing rates of spikelet abortion and pollen sterility (Ji *et al*. 2010; Fakhri *et al*. 2011). However, the effect of pre-anthesis water-deficit stress on grain number has received less attention despite its significant effect on crop yield. Under abiotic stress, grain number is the primary determinant of yield stability because grain number, rather than grain size, mainly accounts for yield loss (Sinclair and Jamieson 2006; Ugarte *et al*. 2007; Dolferus *et al*. 2011). Grain number potential is determined in the early reproductive developmental stages before flowering when water deficiency can affect spike differentiation, or later when water deficiency can cause spikelet or floret abortion (Ugarte *et al*. 2007; Dolferus *et al*. 2011). In Australia, durum wheat is predominantly grown in northern New South Wales, South Australia and western Victoria. In southern Australia, durum wheat production primarily suffers yield loss from lack of rainfall during spring, which causes mild water-deficit stress at pre-anthesis. Lack of spring rain has been shown to lead to a moderate stress (but not severe drought stress) for durum wheat at anthesis, and the stress intensifies through grain filling (French and Schultz 1984*a*, 1984*b*; Rickert *et al*. 1987). Given the limited and unpredictable precipitation that occurs, breeding for genotypes adapted to pre-anthesis water-deficit stress, albeit challenging, is therefore required.

To select adapted genotypes effectively under water-deficit stress, a holistic perception of plant tolerance mechanisms in combination with physiological and morphological responses to water-limited conditions is essential. Under natural field conditions, discriminating between avoidance (e.g. early flowering time to avoid occurrence of water deficiency) and tolerance (e.g. reduced oxidative damage and protected dry-matter accumulation) mechanisms is challenging because of the altered flowering time under stress (Dolferus *et al*. 2011). As a result, crop breeders' efforts

to select optimal stress-tolerant genotypes in the field usually leads to the development of lines with stress avoidance instead of stress tolerance. Stress tolerance, defined agronomically as the ability for a crop to maintain acceptable yield under mild stress (Tardieu and Tuberosa 2010), is therefore difficult to breed for because of the likely exposure of the crop to many and varied stresses in the field. Experiments conducted under controlled glasshouse environments enable precise control of complicated variables and accurate interpretation of the results. Under glasshouse conditions, water-deficit stress treatment can be effectively imposed during booting, usually 10–15 days before anthesis, which is the most important stage for the determination of grain number in cereal crops such as durum wheat. Consequently, a thorough analysis of yield components such as biomass, grain weight and, in particular, grain number under pre-anthesis water-deficit stress will assist durum wheat breeders in Mediterranean conditions to develop a better understanding of stress tolerance and identify adaptive genotypes during breeding.

Studies of morphological and physiological responses under water-deficit stress also contribute greatly to an understanding of the ability of crops to respond and adapt to unfavourable environments. Morphological traits such as plant height, total number of tillers per plant, number of fertile tillers per plant, main spike length, peduncle length and awn length not only affect stress tolerance to limiting soil moisture in cereal crops, they also indicate how adaptive genotypes cope with water shortage via morphological changes (Muhammad and Ihsan 2004; Nouri-Ganbalani *et al*. 2009; Anjum *et al*. 2011; Chen *et al*. 2012). Physiological adaptive responses and indices for evaluating water-deficit response in crops include leaf water potential, chlorophyll content, photosynthetic rate, stomatal conductance, and transpiration rate (Araus *et al*. 2008). Several studies have shown that bread wheat genotypes that are tolerant to water-deficit stress can maintain significantly higher relative water content, chlorophyll content, leaf succulence and cell membrane stability than other genotypes (Sairam and Saxena 2000; Dhanda *et al*. 2004; Praba *et al*. 2009; Arjenaki *et al*. 2012; Shi *et al*. 2014). Varieties maintaining high relative water content or leaf water potential have high turgor potential, which reduces the inhibition of the photosynthetic rate under water deficiency. Chlorophyll content can also be used as a measure of dry-matter accumulation status under oxidative damage caused by water deficiency (Anjum *et al*. 2011). Positive correlations have been found between chlorophyll content and grain yield in bread wheat cultivars under drought (Paknejad *et al*. 2007). Other physiological responses to water deficit vary greatly, depending on the genotypes. For example, genotypic variation in water-deficit tolerance with respect to leaf water potential under water-deficit conditions has been reported in several crops such as bread wheat (Praba *et al*. 2009; Ashraf *et al*. 2013), barley (Vaezi *et al*. 2010), rice (Kamoshita *et al*. 2004), and maize (Efeoğlu *et al*. 2009). However, little is known about the physiological responses under mild water-deficit stress at pre-anthesis, and their correlation with yield in different durum wheat genotypes.

Understanding morphological and physiological response mechanisms under water-deficit stress will assist breeding programs to identify representative traits that are related to yield production. Correlative analysis of those morphological and physiological traits can be used to provide reliable criteria for selecting water-deficiency-tolerant genotypes with improved yield in water-limiting environments (Farshadfar *et al*. 2013). In the very early stages of a breeding program, chlorophyll content has been used as an easy-to-conduct and cost-effective method of indirect selection for improving water-use efficiency in bread wheat (Fotovat *et al*. 2007). However, given the limited knowledge of how the specific yield components and morphological and physiological traits respond to pre-anthesis water stress, this correlative analysis has not been used as a selection tool for durum breeding. Therefore, the objectives of this study were to evaluate the morphological and physiological responses of 20 durum wheat genotypes exposed to moderate pre-anthesis water-deficit stress, and to identify effective morphological or physiological indicators to facilitate a screening process in breeding programs that will lead to enhanced crop yield under water-limiting Mediterranean conditions.

Materials and methods

Plant materials and growth

Twenty durum genotypes (13 varieties and seven University of Adelaide breeding lines) were included in this study (Table 1). The 13 durum varieties were chosen for their known performance under water-limiting conditions or because they are commonly grown in Australia. The seven University of Adelaide durum breeding lines are important advanced entries in Durum Breeding Australia's (DBA) southern-node breeding program; however, their performance under water-limiting environments is unknown. Seeds were obtained from the Australian Winter Cereals Collection (AWCC) or from DBA.

All durum genotypes were grown in a glasshouse on the Waite campus of the University of Adelaide at 22°C−12°C day–night temperature with a 12-h photoperiod. For each genotype, seeds of similar weight were chosen. Seeds were germinated on moist filter paper at room temperature before being transferred to pots (8.5 cm by 8.5 cm by 18 cm) (two seedlings per pot). Seedlings were grown in pots with ~1.2 kg Mt Compass sand containing 0.5% $CaCO_3$. Basal nutrient solution was supplied to all pots during the young seedling stage (growth stage Z10, two leaves emerged; Zadoks *et al.* 1974) and immediately before booting (Z40, flag leaf emergence). The basal solution contained (g L^{-1}): NH_4NO_3 (21), KH_2PO_4 (9), K_2SO_4 (14.4), $MgSO_4 \cdot 7H_2O$ (10.8), $MnSO_4 \cdot H_2O$ (0.84), $CuSO_4 \cdot 5H_2O$ (0.6), H_3BO_3 (0.06), $CoSO_4 \cdot 7H_2O$ (0.12), $FeSO_4 \cdot 7H_2O$ (1.896), $ZnSO_4 \cdot 7H_2O$ (0.12), MoO_3 (0.24), and $NiSO_4 \cdot 6H_2O$ (0.09) (Genc and McDonald 2008).

In 2013, 20 durum genotypes within three treatment groups (control, 12% soil water content (SWC) (equivalent to field capacity); 6% SWC; 4% SWC) were screened for yield components and morphological traits (plant height, fertile tiller number and main spike length) in two experiments (February–July, May–November) with three replicates in each experiment. In 2014, based on the 2013 results, yield components and physiological traits were measured in a subset of genotypes, using only 12% and 6% SWC as treatments and with six replicates. The genotypes used were three varieties sensitive to water-deficit stress (EGA Bellaroi, Tjilkuri and Caparoi), three tolerant varieties (Tamaroi, Yawa and WID802), and one DBA line of interest (DBA-Aurora, which was released as a variety in spring 2014) (Table 2).

Table 1. Durum genotypes used in this study with their origins and sensitivity to water-deficit stress (if known)
UAD, University of Adelaide durum breeding line

Variety or line	Origin	Sensitivity to water-deficit stress (if known) and other comments
EGA Bellaroi	Australia	New South Wales variety, grown for its quality, unknown sensitivity
Caparoi	Australia	New South Wales variety, unknown sensitivity
Cham 1	Unknown	High yield stability under water-limiting environment (Pecetti and Annicchiarico 1993)
DBA-Aurora	Australia	Evaluated as UAD0951096, high yield potential, released as DBA-Aurora in spring 2014, unknown sensitivity
Jandaroi	Australia	New South Wales variety maturing earlier than other New South Wales varieties, unknown sensitivity
Nelly-1	Mexico	High grain yield and high 1000-grain weight under drought conditions (Arzani 2002)
Omrabi-3	Syria	Grain yield intermediate with lower number kernels per spike and kernel weight (Garcia del Moral *et al*. 2003)
Saintly	Australia	South Australian variety maturing earlier than other varieties, unknown sensitivity
Tamaroi	Australia	South Australian variety, unknown sensitivity
Tjilkuri	Australia	Adapted South Australian variety, moderate early vigour, unknown sensitivity
Waha	Algeria	Yield component reduction ~30% as a result of water deficit stress (Fakhri *et al*. 2011)
WID802	Australia	Adapted South Australian variety, moderate early vigour, unknown sensitivity
Yawa	Australia	Adapted South Australian variety, high yield potential, unknown sensitivity
UAD1053255	Australia	Unknown sensitivity
UAD1151112	Australia	Unknown sensitivity
UAD1152081	Australia	Unknown sensitivity
UAD1153124	Australia	Unknown sensitivity
UAD1153173	Australia	Unknown sensitivity
UAD1153177	Australia	Unknown sensitivity
UAD1153303	Australia	Unknown sensitivity

Treatments to induce water-deficit stress

All treatment groups were well watered to field capacity (12% SWC) from germination to booting stage. When the sheath of the flag leaf extended at the start of booting stage (Z43), water-limiting stress treatments were imposed. SWC was maintained at 12% from booting to harvest in the control group and at 6% (50% of field capacity) and 4% (33% of field capacity) in each of two water-deficit stress groups from booting to harvest. SWC was monitored daily by weighing the pots each morning. Watering was applied when necessary to maintain SWC, ensuring controlled water availability for the plants. The 6% SWC treatment is considered moderate water-deficit stress, similar to water availability during spring in a rainfed environment in South Australia. The 4% SWC treatment is considered severe water-deficit stress, similar to drought conditions (Praba *et al*. 2009; Akhkha *et al*. 2011; Ashinie *et al*. 2011).

Responses to water-deficit stress

Relative water content (RWC), leaf water potential, and chlorophyll content were measured after 15 days of water-deficit stress. RWC was measured on the penultimate leaf. Fresh leaves were taken from each plant after 15 days of water-deficit stress and weighed immediately to record fresh weight (FW). Leaves were then placed in distilled water for 5 h and weighed again to record turgid weight (TW). Dry weight (DW) was recorded after oven drying at 70°C for 24 h. RWC was estimated using the formula: (FW – DW)/(TW – DW) × 100 (Barrs and Weatherley 1962). Flag leaf water potential was measured with a pressure chamber (PMS Instruments, Corvallis, OR, USA). Measurements of chlorophyll content were made three times along the middle section of the flag leaf with a chlorophyll meter (SPAD-502; Konica Minolta, Osaka) and the mean value used for analysis was listed as SPAD units.

At the end of the growth period, plants were harvested from each pot. Grain weight per plant, number of grains per plant, biomass, plant height, number of fertile tillers per plant, and main spike length were determined. Plant height was obtained by measuring from the base of the stem to the tip of the spike

Table 2. Rank summation index of 20 durum genotypes for their sensitivity to pre-anthesis water deficit
The scores of genotypes under each yield component are given based on their ranking in the yield component when under moderate stress (6% soil water content) relative to the control (using data shown in Figs 1*b*, 2*b*, 3*b*, 4*b*). Genotypes with lower rankings are more sensitive to water-deficit stress, and those with higher rankings are more tolerant. Chosen for the 2014 experiment: EGA Bellaroi, Tjilkuri and Caparoi as genotypes sensitive to water-deficit stress; DBA-Aurora as the Durum Breeding Australia line of interest; WID802, Yawa and Tamaroi as genotypes tolerant to water-deficit stress

Genotype	Grain weight	Biomass	Harvest index	No. of grains	Rank summation index
EGA Bellaroi	2	4	2	1	9
Tjilkuri	4	3	5	4	16
Caparoi	1	12	1	2	16
Nelly-1	3	1	20	3	27
UAD1152081	5	14	3	6	28
Cham-1	16	5	6	7	34
DBA-Aurora	6	15	7	8	36
UAD1053255	12	2	11	13	38
UAD1151112	8	8	13	10	39
UAD1153124	7	11	4	11	39
Jandaroi	10	6	16	9	41
UAD1153117	9	10	18	5	42
UAD1153303	11	11	8	15	45
Omrabi-3	14	9	19	12	54
Waha	15	13	10	16	54
UAD1153173	17	7	17	14	55
Saintly	13	18	9	17	57
WID802	18	19	12	18	67
Yawa	19	16	15	20	70
Tamaroi	20	20	14	19	73

(main stem, awns not included). Main spike length was measured on the main stem from the base of the first spikelet to the tip of the last spikelet (awns not included). Harvest index was calculated as the ratio of grain dry weight to biomass (Donald 1962). Grain weight per plant, grain number, and biomass were determined relative to the control using the following equation: (mean value of water-deficit group – mean value of control group)/mean value of control group × 100.

Statistical analyses

Regardless of experiment, pots were arranged randomly in the glasshouse. To determine sensitivity to water-deficit stress based on Fischer and Maurer (1978), yield components for the control group and water-deficit-stress groups were compared for each individual genotype. Significant changes in morphological and physiological traits in response to water-deficit stress were also identified for each individual genotype. One-way analyses of variance (ANOVA) were performed for the 2013 data and Student's *t*-tests for the 2014 data, using GENSTAT 15th edition (VSN International Ltd, Hemel Hempstead, UK). Where appropriate, means among treatment groups were compared using least significant difference at $P = 0.05$ to detect significance. Correlation coefficients were also calculated for all yield-component combinations.

Results

Is the effect of pre-anthesis water deficit on yield components genotype-dependent?

In the 2013 experiments, for most of the 20 durum wheat genotypes, grain weight and biomass were significantly reduced ($P < 0.05$) under water-deficit-stress treatments (6% SWC and 4% SWC) compared with the control treatment (12% SWC) (Figs 1*a*, 2*a*). Under both water-deficit-stress treatments, grain weight and biomass relative to the control differed among genotypes (Figs 1*b*, *c*, 2*b*, *c*). Genotypes with a relatively small reduction in both grain weight and biomass are tolerant to water-deficit stress (e.g. Tamaroi, Yawa and WID802), whereas genotypes with a relatively large reduction in both grain weight and biomass are sensitive to water-deficit stress (e.g. EGA Bellaroi, Tjilkuri and Caparoi). For each genotype, the reductions in grain weight and biomass relative to the control group under the two levels of water-deficit stress were different (Figs 1*b*, *c*, 2*b*, *c*). For example, reductions in grain weight of Tamaroi were smaller than of other genotypes evaluated under the two water-deficit-stress treatments (Fig. 1*b*, *c*). The reductions in grain weight and biomass of EGA Bellaroi were large under both water-deficit-stress treatments (Figs 1*b*, *c*, 2*b*, *c*). By contrast, Yawa showed a small reduction in grain weight and biomass under 6% SWC but relatively large reduction under 4% SWC (Figs 1*b*, *c*, 2*b*, *c*). Similarly, DBA-Aurora showed a small reduction in biomass under 6% SWC but a relatively large reduction under 4% SWC (Fig. 2*b*, *c*).

No significant difference ($P > 0.05$) in harvest index was observed in any genotypes between both water-deficit-stress treatments and the control treatment (Fig. 3*a*). However, under both water-deficit-stress treatments, the relative harvest index showed differences among genotypes (Fig. 3*b*, *c*). Varieties such as EGA Bellaroi and Caparoi showed lower harvest index under water-deficit stress, whereas genotypes such as UAD1151112 had a higher harvest index when placed under water-deficit stress (Fig. 3*b*, *c*).

In the 2013 experiments, the grain number of 12 genotypes was significantly reduced ($P < 0.05$) under both water-deficit-stress treatments compared with the control (Fig. 4*a*). Under both water-deficit-stress treatments, the grain number relative to the control differed among genotypes (Fig. 4*b*, *c*). Genotypes with no significant reduction in grain number are tolerant to water-deficit stress (e.g. Tamaroi, Yawa and WID802), and genotypes with a significant, large reduction in grain number are sensitive to water-deficit stress (e.g. EGA Bellaroi, Tjilkuri and Caparoi). For each genotype, the reduction in grain number relative to the control group differed between the two levels of water-deficit stress (Fig. 4*b*, *c*). For example, the reduction in grain number of Tamaroi was small compared with other genotypes under the two water-deficit-stress treatments (Fig. 4*b*, *c*). By contrast, the genotype Yawa showed a relatively small reduction in grain number under 6% SWC but a relatively large reduction under 4% SWC (Fig. 4*b*, *c*).

From the initial 20 durum genotypes evaluated, three varieties sensitive to water-deficit stress (EGA Bellaroi, Tjilkuri and Caparoi), three tolerant varieties (Tamaroi, Yawa and WID802), and one DBA line of interest (DBA-Aurora) were chosen for further experimentation (Fig. 5), based on their yield performance in response to the 6% SWC treatment (Table 2). Grain weight, grain number, biomass and harvest index were used to evaluate sensitivity to water-deficit stress, because grain weight had strong positive correlations with grain number ($r = 0.91$), biomass ($r = 0.79$) and harvest index ($r = 0.82$) (Table 3). The rank summation index (Table 2) for the water-deficit-stress sensitivity of each yield component is given based on the ranking of each genotype in terms of yield component loss under the 6% SWC treatment relative to the control (Figs 1*b*, 2*b*, 3*b* and 4*b*). Genotypes with a higher rank summation index are more tolerant to water-deficit stress. Under moderate water-deficit stress, compared with the control treatment, sensitive varieties showed reduced leaf greenness and biomass (Fig. 5*a*), whereas tolerant varieties (Fig. 5*b*) and DBA-Aurora (Fig. 5*c*) appeared similar to the control.

In the 2014 experiment, of the seven selected durum wheat genotypes, the three varieties sensitive to water-deficit stress (EGA Bellaroi, Tjilkuri and Caparoi) showed significant reductions similar to the first experiment (2013) for grain weight, biomass and grain number under 6% SWC ($P < 0.05$) (Table 4). Similarly, the three varieties tolerant to water-deficit stress (Tamaroi, Yawa and WID802) had no significant reduction in grain weight, biomass and grain number under water-deficit stress ($P < 0.05$) (Table 4). The harvest index of all seven varieties showed no significant change under water-deficit stress ($P < 0.05$) (Table 4).

Is the effect of pre-anthesis water deficit on morphological traits genotype-dependent?

In the 2013 experiment, plant height of durum genotypes was generally reduced under water-deficit-stress conditions compared with the control (Fig. 6*a*). Significant reductions in plant height ($P < 0.05$) were observed in five genotypes (DBA-Aurora,

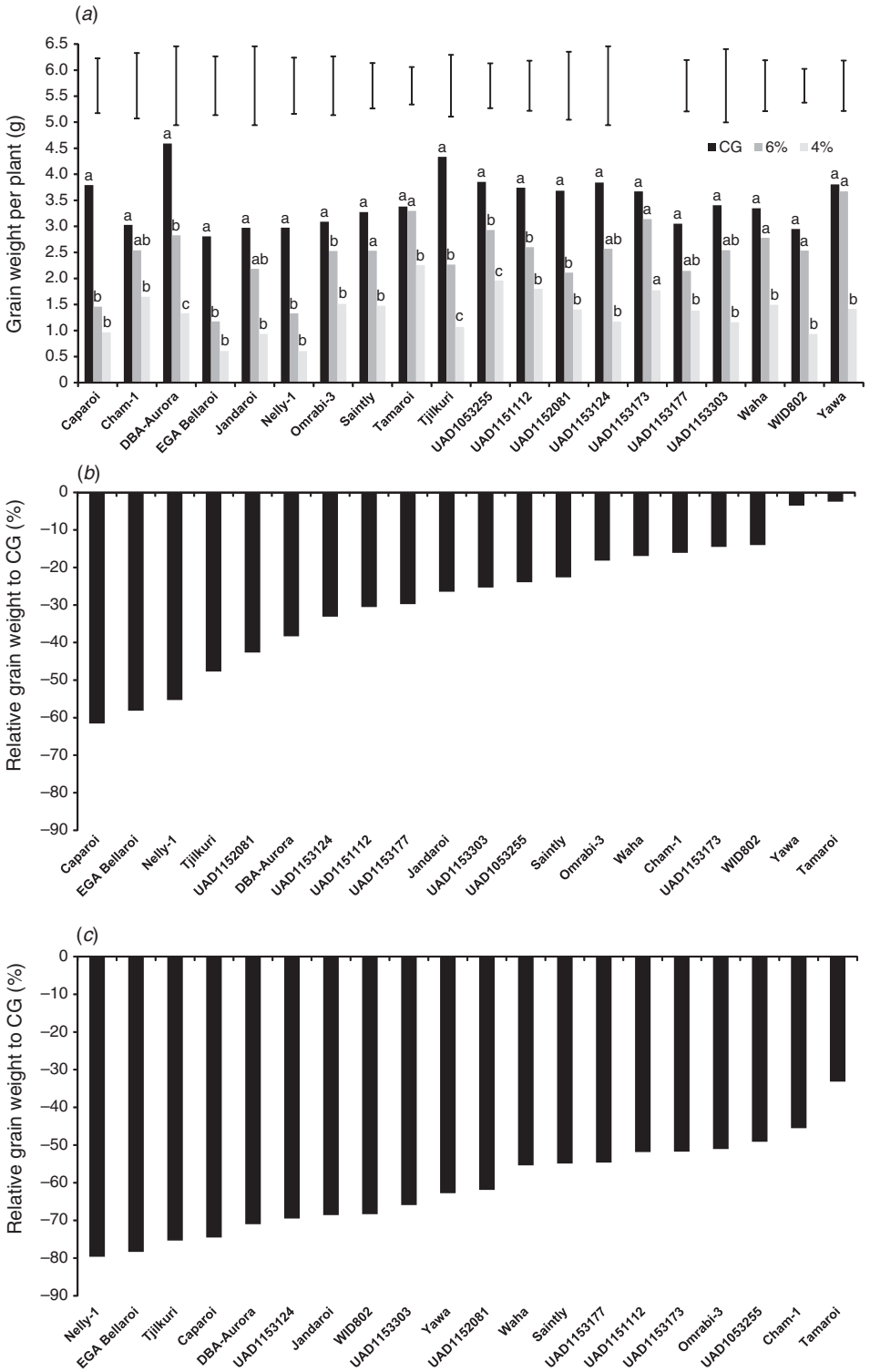

Fig. 1. Effect of pre-anthesis water deficit on grain weight in durum wheat genotypes. Between booting and harvest, genotypes were grown under 12% soil water content (SWC) (field capacity, control), 6% SWC (moderate water-deficit stress) or 4% SWC (severe water-deficit stress). (*a*) Grain weight per plant was measured at harvest and used to determine the relative grain weight (relative to the control) for (*b*) 6% SWC and (*c*) 4% SWC. Relative grain weights are shown in descending order of effect of water deficit, with the most sensitive genotypes on the left and the most tolerant on the right. Means are shown for $n = 6$ from two experiments in 2013. Capped lines are l.s.d. ($P = 0.05$) for comparison among treatments for each genotype, and means with the same letter are not significantly different.

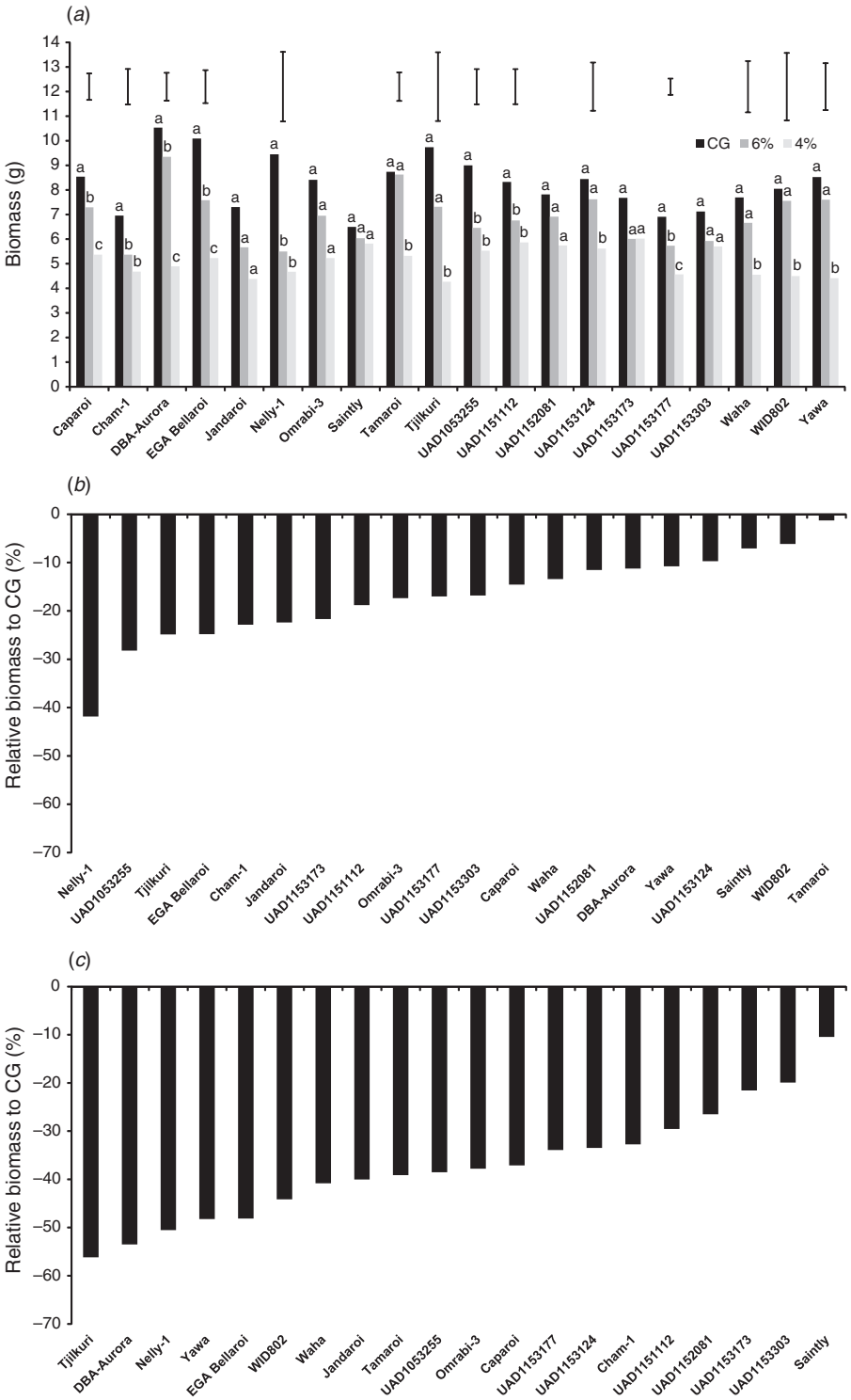

Fig. 2. Effect of pre-anthesis water deficit on biomass in durum wheat genotypes. Between booting and harvest, genotypes were grown under 12% soil water content (SWC) (field capacity, control), 6% SWC (moderate water-deficit stress) or 4% SWC (severe water-deficit stress). (*a*) Biomass was measured at harvest and used to determine the relative biomass (relative to the control) for (*b*) 6% SWC and (*c*) 4% SWC. Relative biomass is shown in descending order of effect of water deficit, with the most sensitive genotypes on the left and the most tolerant genotypes on the right. Means are shown for $n = 3$ from one representative experiment in 2013. Capped lines are l.s.d. ($P = 0.05$) for comparison among treatments for each genotype, and means with the same letter are not significantly different.

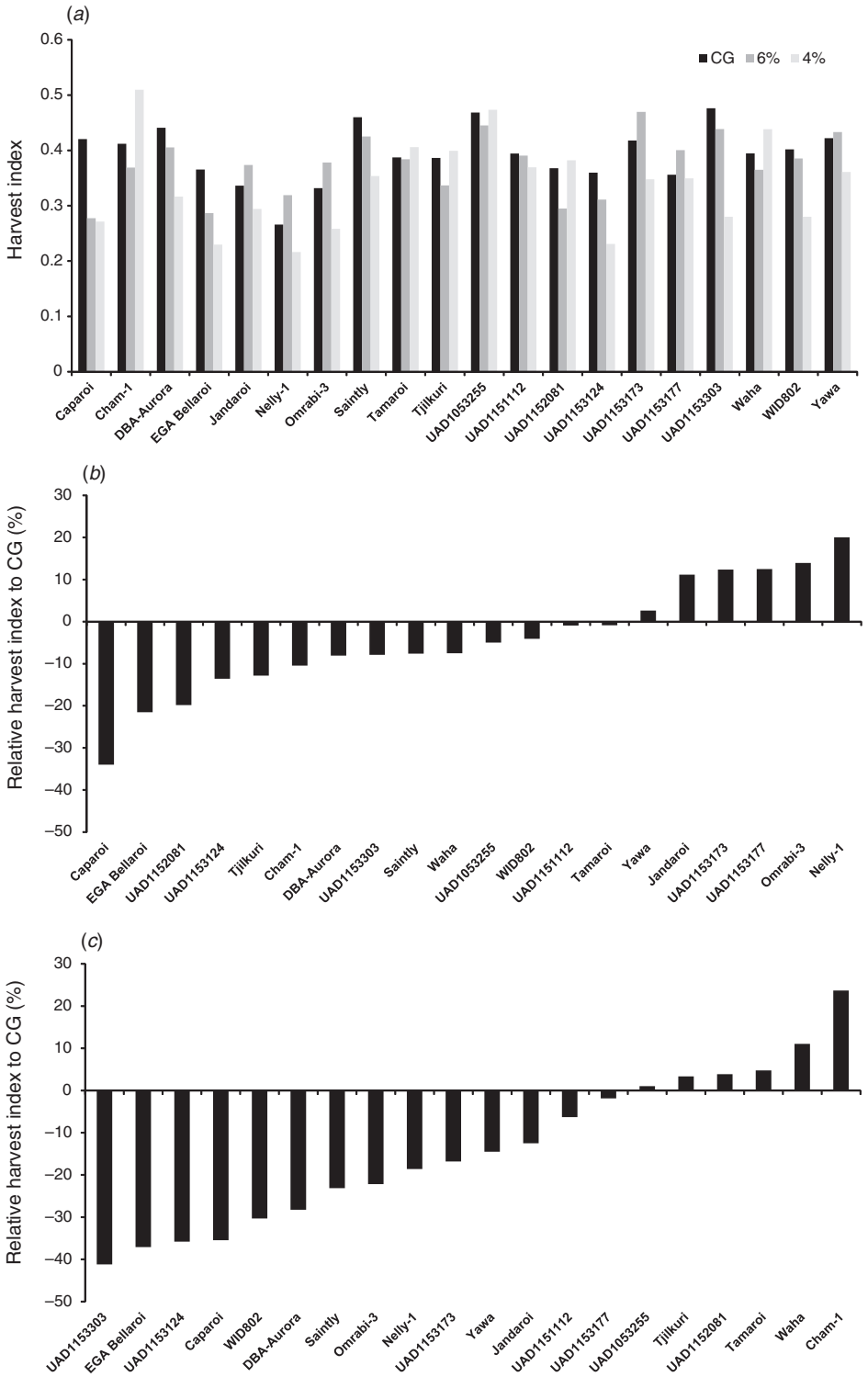

Fig. 3. Effect of pre-anthesis water deficit on harvest index in durum wheat genotypes. Between booting and harvest, genotypes were grown under 12% soil water content (SWC) (field capacity, control), 6% SWC (moderate water-deficit stress) or 4% SWC (severe water-deficit stress). (*a*) Harvest index was measured at harvest and used to determine the relative harvest index (relative to the control) for (*b*) 6% SWC and (*c*) 4% SWC. Relative harvest index is shown in descending order of effect of water deficit, with the most sensitive genotypes on the left and the most tolerant genotypes on the right. Means are shown for $n = 3$ from one representative experiment in 2013. There were no significant differences ($P > 0.05$) between means for any of the genotypes.

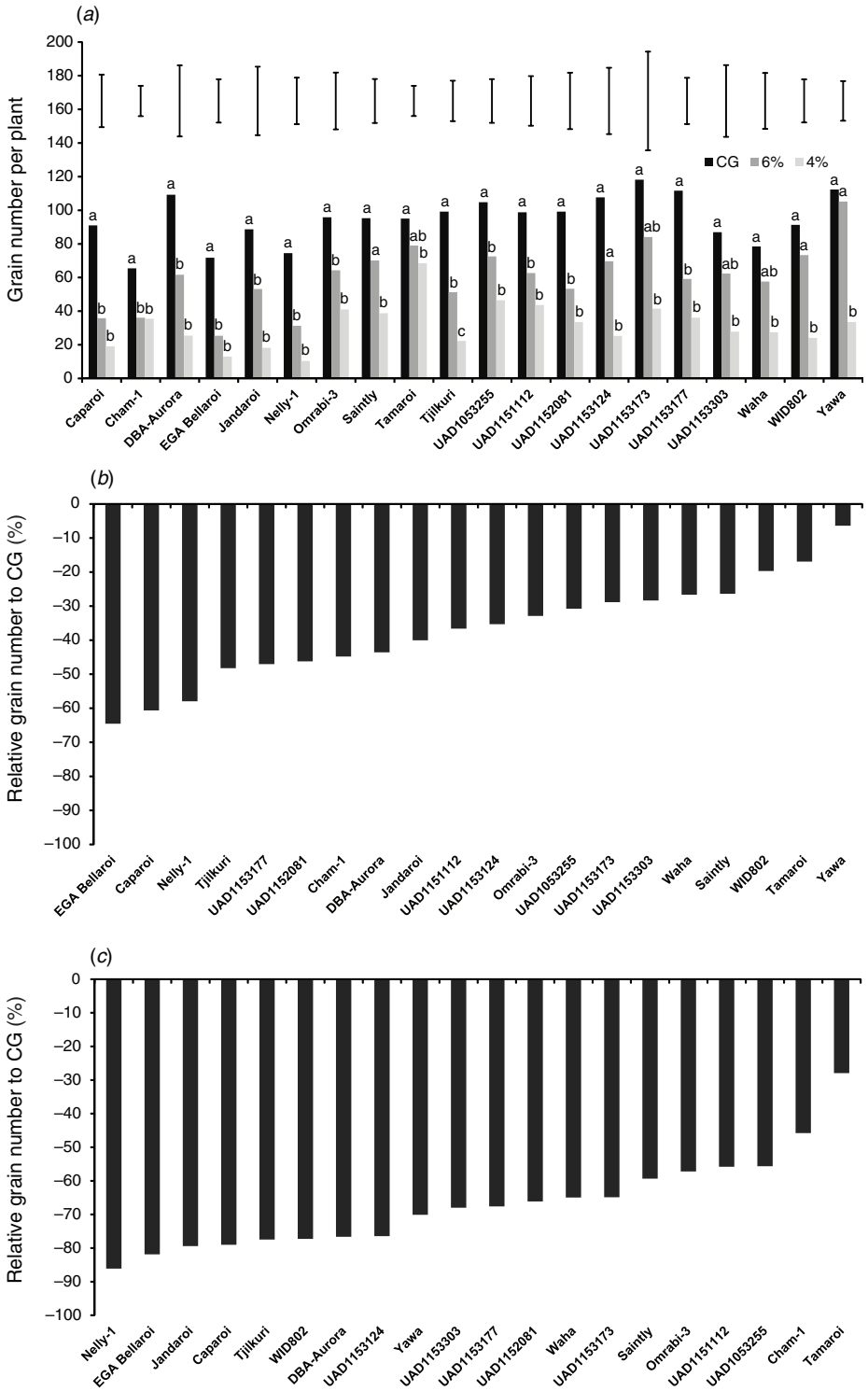

Fig. 4. Effect of pre-anthesis water deficit on number of grains in different durum wheat genotypes. Between booting and harvest, genotypes were grown under 12% soil water content (SWC) (field capacity, control), 6% SWC (moderate water-deficit stress) or 4% SWC (severe water-deficit stress). (*a*) Grain number was measured at harvest and used to determine the relative grain number (relative to the control) for (*b*) 6% SWC and (*c*) 4% SWC. Relative grain numbers are shown in descending order of effect of water deficit, with the most sensitive genotypes on the left and the most tolerant genotypes on the right. Means are shown for $n = 6$ from two experiments in 2013. Capped lines are l.s.d. ($P = 0.05$) for comparison among treatments for each genotype, and means with the same letter are not significantly different.

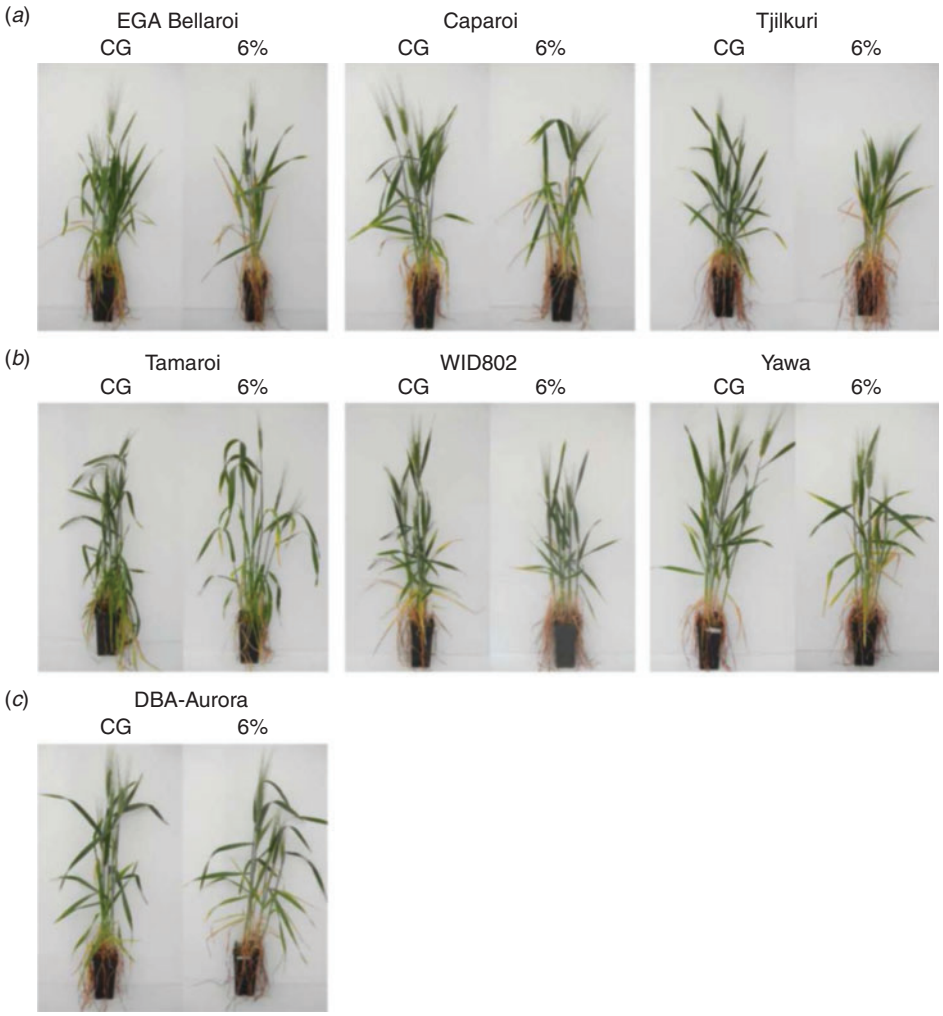

Fig. 5. Variety differences between the seven selected durum wheat genotypes under a moderate (6% soil water content, SWC) water-deficit-stress treatment. (*a*) Genotypes sensitive to water-deficit stress, (*b*) tolerant genotypes, (*c*) Durum Breeding Australia line of interest (DBA-Aurora). After 15 days of water-deficit stress, Tamaroi, WID802 and Yawa continued to grow relatively well compared with EGA Bellaroi, Caparoi and Tjilkuri. CG, Control group (12% SWC, field capacity).

Table 3. Correlation coefficients between yield components and morphological traits in 20 durum wheat genotypes
Strong correlation indicated in **bold**; weak to moderate correlation in *italics*

	No. of grains per plant	1000-grain weight	Biomass	Harvest index	Plant height	No. of tillers per plant	Main spike length
Grain weight per plant	**0.91**	–0.20	**0.79**	**0.82**	0.39	0.30	0.06
No. of grains per plant		*–0.51*	**0.74**	**0.73**	*0.41*	*0.36*	0.06
1000-grain weight			–0.15	–0.17	–0.16	–0.21	0.03
Biomass				0.33	*0.47*	*0.44*	0.11
Harvest index					0.19	0.11	–0.03
Plant height						0.04	0.25
No. of tillers per plant							–0.21

Omrabi-3, UAD1053255, UAD1152081 and Waha) between the control and 6% SWC treatment, and in four genotypes (Jandaroi, Nelly-1, UAD1153124 and Yawa) between 6% and 4% SWC (Fig. 6*a*).

Fertile tiller number of durum genotypes was generally reduced under water-deficit stress (Fig. 6*b*). Six genotypes (Cham-1, Jandaroi, Nelly-1, UAD1151112, Waha and Yawa) had significant reductions ($P < 0.05$) in fertile tiller number under

Table 4. Yield components per plant in seven durum genotypes under field capacity [12% soil water content (SWC), control] and moderate water-deficit stress (6% SWC) in 2014

Means ± s.e. are shown for $n = 6$. *Indicates a significant ($P < 0.05$) difference between the control and 6% SWC treatment for each genotype, as determined by *t*-test

Variety	Grain weight (g)		Biomass (g)		Harvest index		No. of grains	
	12% SWC	6% SWC	12% SWC	6% SWC	12% SWC	6% SWC	12% SWC	6% SWC
EGA Bellaroi	1.02 ± 0.05	0.71 ± 0.09*	3.64 ± 0.18	2.69 ± 0.19*	0.28 ± 0.01	0.26 ± 0.03	24.3 ± 1.4	17.3 ± 2.2*
Tjilkuri	1.52 ± 0.15	1.05 ± 0.07*	4.53 ± 0.32	2.98 ± 0.20*	0.33 ± 0.02	0.36 ± 0.02	42.3 ± 3.4	25.5 ± 3.0*
Caparoi	1.09 ± 0.11	0.79 ± 0.11*	3.34 ± 0.20	2.35 ± 0.34*	0.32 ± 0.03	0.36 ± 0.04	25.0 ± 2.1	16.7 ± 2.2*
Tamaroi	1.38 ± 0.08	1.25 ± 0.09	3.92 ± 0.14	3.54 ± 0.10	0.35 ± 0.02	0.35 ± 0.02	36.8 ± 3.7	31.8 ± 1.6
Yawa	1.53 ± 0.13	1.33 ± 0.06	3.62 ± 0.20	3.16 ± 0.12	0.41 ± 0.01	0.42 ± 0.01	43.5 ± 3.3	37.3 ± 1.9
WID802	1.37 ± 0.05	1.13 ± 0.09	3.39 ± 0.18	2.96 ± 0.12	0.41 ± 0.02	0.38 ± 0.01	40.8 ± 2.7	33.5 ± 2.8
DBA-Aurora	1.81 ± 0.13	1.41 ± 0.12*	4.37 ± 0.34	3.38 ± 0.19*	0.42 ± 0.02	0.42 ± 0.03	45.0 ± 2.2	31.2 ± 2.6*

both water-deficit-stress treatments. Six genotypes (Caparoi, Tjilkuri, UAD1053255, UAD1153124, UAD153177 and UAD1153303) also displayed significant reductions ($P < 0.05$) in fertile tiller number between 6% and 4% SWC. EGA Bellaroi was the only genotype in which fertile tiller number was significantly reduced ($P < 0.05$) from the control to 6% SWC and again from 6% to 4% SWC.

No significant difference ($P > 0.05$) in main spike length was observed for any genotype between the control and water-deficit-stress treatments (Fig. 6*c*). However, compared with the other 17 genotypes, three genotypes (UAD1152081, Waha and WID802) displayed a trend for spike length to increase as water supply became more limiting. By contrast, EGA Bellaroi, Tjilkuri and UAD1153177 tended to show reduced main spike length under both water-deficit stress treatments compared with the control.

Of the morphological traits evaluated, correlations of plant height with yield components were weak to moderately positive for biomass ($r = 0.4712$) and grain number ($r = 0.4107$), whereas fertile tiller number had a weak to moderate positive correlation with biomass ($r = 0.4391$) and grain number ($r = 0.3582$) (Table 3).

Do water deficit stress-sensitive and stress-tolerant genotypes have distinct response patterns for certain physiological traits?

In the 2014 experiment, the chlorophyll content of all seven selected durum genotypes decreased under water-deficit stress (Table 5). Three genotypes sensitive to water-deficit stress, EGA Bellaroi, Tjilkuri and Caparoi, showed significant reductions in chlorophyll content of 12.3%, 9.2% and 10.4%, respectively, whereas the three genotypes tolerant to water-deficit stress, Tamaroi, Yawa and WID802, showed no significant reduction. DBA-Aurora had a low–moderate significant reduction in chlorophyll content (5.5%).

Leaf relative water content was generally reduced under the water-deficit-stress treatment compared with the control (Table 5). Significant reductions in relative water content were observed in EGA Bellaroi, Tjilkuri, Caparoi, Tamaroi and DBA-Aurora under pre-anthesis water-deficit stress. No significant reduction was observed in the tolerant genotypes Yawa and WID802. Of the durum genotypes evaluated, DBA-Aurora recorded the largest reduction in relative water content, and WID802 the smallest reduction.

Significant ($P < 0.05$) reductions in leaf water potential were observed in all seven durum genotypes due to pre-anthesis water deficit stress (Table 5). However, reductions were more pronounced in the genotypes sensitive to water-deficit stress (EGA Bellaroi, Tjilkuri and Caparoi). The largest reduction in leaf water potential was recorded in Caparoi and the smallest in Yawa.

For the physiological traits measured, a weak to moderate positive correlation was observed between chlorophyll content and grain weight ($r = 0.5550$) (Fig. 7*a*), whereas leaf water potential was found to be moderately negatively correlated with grain weight ($r = –0.6178$) (Fig. 7*b*).

Discussion

In the present study, genotypic differences in response to pre-anthesis water-deficit stress in 20 durum wheat genotypes were assessed on the basis of yield components, morphological traits and physiological traits. Water-deficit stress causes reductions in grain weight per plant, number of grains per plant, biomass, plant height, and number of fertile tillers in all genotypes, with considerable variations observed in the set of durum wheat genotypes studied. The response to pre-anthesis water-deficit stress in harvest index and main spike length varied across 20 different durum wheat genotypes. Among all yield responses to pre-anthesis water-deficit stress, grain number was the most affected yield component.

Although controlled glasshouse conditions provide many benefits with respect to screening genotypes for their responses to a specific stress, there can be limitations in extrapolating yield performance from pots to the field (Poorter *et al*. 2012; Rebetzke *et al*. 2014). The use of small pots or plot size might lead to biological constraints of biomass and yield due to resource competition between plots, subsequently affecting genotypes in various ways (Poorter *et al*. 2012; Rebetzke *et al*. 2014). Phenotyping of stress-tolerance improvement in controlled experiments cannot simply depend on direct selection for high grain yield under water-stressed conditions across different genotypes, but must incorporate evaluation of yield reduction caused by the stress within each genotype. Target traits to achieve yield stability, such as the maintenance of grain number and floral fertility despite water-deficit stress, are therefore useful indicators. In this study, three varieties (EGA Bellaroi, Tjilkuri and Caparoi) with most significant reductions in grain weight per plant, grain number and biomass were considered sensitive to

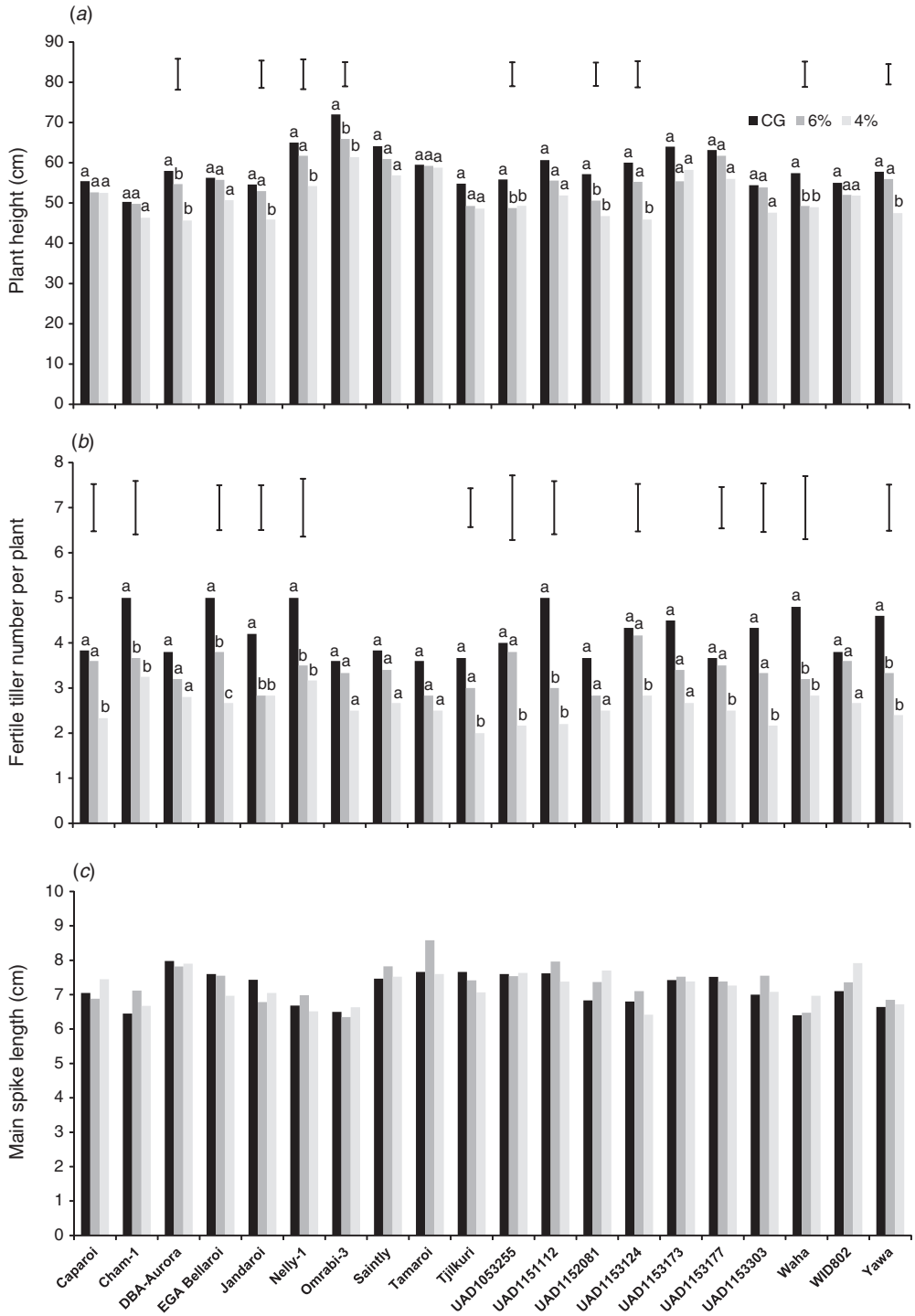

Fig. 6. Effect of pre-anthesis water deficit on morphological traits in different durum wheat genotypes. Between booting and harvest, genotypes were grown under 12% SWC (field capacity, control), 6% SWC (moderate water-deficit stress) or 4% SWC (severe water-deficit stress). (*a*) Plant height, (*b*) tiller number and (*c*) main spike length were measured at harvest. Means are shown for $n = 6$ from two experiments in 2013. Capped lines are l.s.d. ($P = 0.05$) for comparison among treatments for each genotype, and means with the same letter are not significantly different.

water-deficit stress. Three varieties (Tamaroi, Yawa and WID802) with the smallest reduction in grain weight per plant, grain number and biomass among all genotypes were considered tolerant of water-deficit stress. Water deficit tolerant or sensitive varieties were chosen based on their response under 6% SWC. Observed genotypic differences in water-deficit stress tolerance

Table 5. Effect of pre-anthesis water-deficit stress on chlorophyll content, leaf water potential and relative water content (RWC) in seven durum genotypes in 2014

Means ± s.e. are shown for $n = 6$. *Indicates a significant ($P < 0.05$) difference between the 12% soil water content (SWC) control treatment and the moderate water deficit stress treatment (6% SWC) for each genotype, as determined by *t*-test

Genotype	Chlorophyll content (SPAD units)		RWC (%)		Leaf water potential (bars)	
	12% SWC	6% SWC	12% SWC	6% SWC	12% SWC	6% SWC
EGA Bellaroi	49.5 ± 0.9	43.4 ± 1.0*	93.7 ± 0.7	88.4 ± 0.9*	4.1 ± 0.2	7.2 ± 0.3*
Tjilkuri	50.4 ± 1.0	45.8 ± 0.4*	96.2 ± 0.9	88.4 ± 1.5*	4.0 ± 0.3	7.1 ± 02*
Caparoi	49.8 ± 0.6	44.6 ± 0.5*	95.0 ± 0.8	89.9 ± 0.7*	3.7 ± 0.1	6.8 ± 0.2*
Tamaroi	48.9 ± 0.6	47.2 ± 0.7	93.6 ± 1.3	85.9 ± 1.2*	3.6 ± 0.2	5.7 ± 0.3*
Yawa	49.1 ± 0.7	47.4 ± 0.3	89.8 ± 0.7	87.0 ± 2.6	3.6 ± 0.1	5.2 ± 0.2*
WID802	49.7 ± 0.2	48.6 ± 0.6	94.4 ± 0.9	91.9 ± 0.7	4.1 ± 0.1	6.6 ± 0.3*
DBA-Aurora	53.2 ± 0.4	50.3 ± 0.5*	94.2 ± 1.0	86.0 ± 2.3*	3.1 ± 0.2	5.7 ± 0.2*

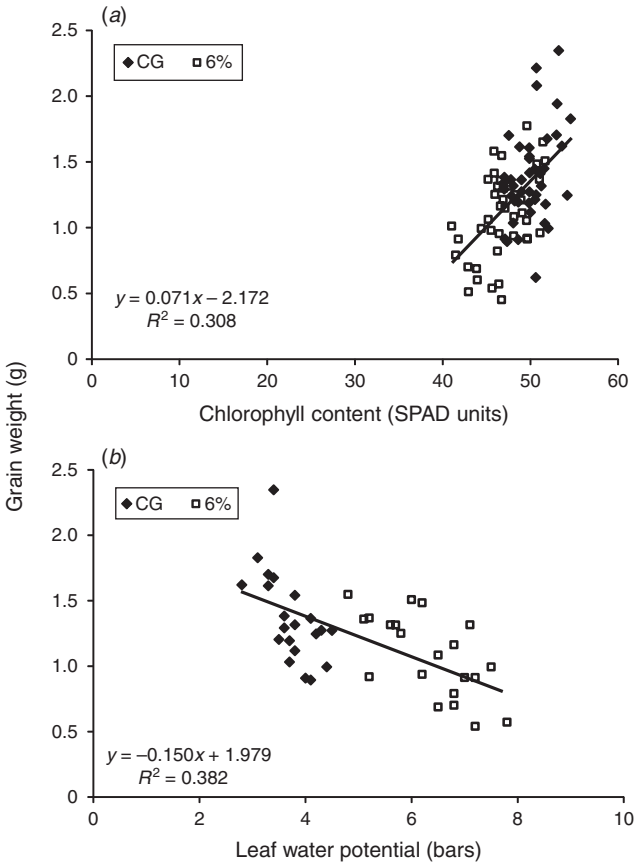

Fig. 7. Associations between grain weight and (*a*) chlorophyll content and (*b*) leaf water potential under the effect of pre-anthesis water deficit in seven durum genotypes in the 2014 experiment. Chlorophyll content and leaf water potential were measured on the flag leaf at 15 days after the booting stage in both the 12% soil water content (SWC) control treatment and the 6% SWC water-stress treatment of each genotype.

based on grain weight per plant in durum wheat genotypes may be due to variations in different morphological and physiological responses. These include the strength of photosynthetic tissues, leaf water status and osmotic adjustment, which substantially contribute to crop growth and productivity (Gupta *et al*. 2001; Guóth *et al*. 2009; Lopes *et al*. 2012).

In the present study, a strong positive correlation between grain number and grain weight per plant ($r = 0.91$) was observed, suggesting that grain number could be a reliable indicator of pre-anthesis water-deficit-stress tolerance across durum genotypes. Under pre-anthesis water-deficit stress, the reduction in grain number, rather than the loss in grain size, was mainly responsible for reduction in grain weight per plant. Grain number in bread wheat has been reported as the primary component contributing to increased yield, and the most affected yield component under water-deficit stress when grain size and harvest index remain unchanged or are reduced (Dolferus *et al*. 2011; Chen *et al*. 2012). Reproductive development stages in self-fertilising cereal crops (e.g. durum wheat, bread wheat, and barley) are extremely susceptible to environmental stress (Barnabas *et al*. 2008; Dolferus *et al*. 2011). The reduction in grain number is the direct result of floral abnormalities, low pollen viability, and pollination inhibition caused by water deficit during booting, floral initiation and differentiation, and anthesis stages, which will ultimately lead to grain yield loss (Solomon *et al*. 2003; Dolferus *et al*. 2011). In this study, durum genotypes better adapted to water-deficit stress were able to maintain their grain number in unfavourable conditions, which contributes to a smaller reduction in grain yield. The ability to maintain grain number and yield stability under water-deficit stress is one of the most important breeding goals for adaptation to water-limiting Mediterranean environments.

In this study, plant height and fertile tiller number were reduced in response to water-deficit stress. A reduction in plant height may be attributed to cell enlargement and cell division inhibition and a higher rate of leaf senescence, which are associated with reduced turgor potential and protoplasm dehydration caused by water-deficit stress (Anjum *et al*. 2011; Khakwani *et al*. 2012). Positive correlations between plant height and biomass, and plant height and grain weight per plant, revealed that plant height is important to the maintenance of straw yield and may have positive effects on the improvement of grain yield. Tiller formation and the maintenance of number of fertile tillers are linked to a high photosynthetic rate and high stomata conductance, which ultimately contributes to shoot biomass (Munns *et al*. 2010). In the present study, the positive correlation of fertile tiller number with biomass is more pronounced than other yield components. The maintenance of grain-bearing tillers until maturity is therefore a good indicator of

uninterrupted photosynthetic activity and an important attribute contributing to biomass production under water-deficit stress.

Adaptive morphological traits (plant height, spike length and fertile tiller number) contribute greatly to yield performance under water-limiting conditions. The correlations reported among morphological traits and yield components made it possible to identify adapted genotypes. Ultimately, the development and release of durum varieties with good agronomic adaptation to rainfed conditions similar to those experienced in South Australia will lead to improved genetic gain and support industry expansion for durum wheat.

Chlorophyll content can directly determine the photosynthetic rate and reflect photosynthetic potential and primary production (Richardson *et al*. 2002; Anjum *et al*. 2011). Reductions in chlorophyll content related to abiotic stress and senescence indicate low concentrations of photosynthetic pigments, which will cause inactivation of photosynthesis, and inhibition of photosynthetic potential and primary production (Anjum *et al*. 2011; Loutfy *et al*. 2012). In this study, chlorophyll content was significantly reduced only in the genotypes sensitive to water-deficit stress. Genotypes with greater tolerance may therefore prevent chlorophyll loss and subsequent impairment of photosynthetic capability when water availability is limited. This is in agreement with a study in bread wheat where chlorophyll content was used as a reliable indicator for evaluating the integrity of the photosynthetic apparatus under stress, and as a selective tool for higher grain yield potential under drought conditions (Abdipur *et al*. 2013). Therefore, the measurement of chlorophyll content by a non-destructive, efficient and reliable approach such as the SPAD meter may be suitable for detecting and quantifying pre-anthesis water-deficit-stress tolerance. Further investigation of photosynthetic activity under water-limiting environments could be enhanced by evaluating gas exchange measurements such as stomatal conductance (Flexas *et al*. 2004; Long *et al*. 2004, 2006). Under water-deficit stress, the inhibition of photosynthesis in C_3 plants such as durum wheat is conditioned by stomatal and non-stomatal limitations (Flexas and Medrano 2002; Long *et al*. 2004).

Maintenance of the appropriate plant water status during water-deficit stress is essential for plant growth and productivity. In this study, water status of durum wheat genotypes was evaluated by determining leaf water potential and relative water content. Leaf water potential and relative water content have both been reported to be reliable parameters for quantifying plant water-stress response. Significant differences of leaf water potential in response to water shortage have been observed among durum wheat and bread wheat cultivars (Subrahmanyam *et al*. 2006; Praba *et al*. 2009; Ashinie *et al*. 2011). The changes in plant water potential might be attributable to a change in osmotic activity. The differences in relative water content between genotypes tolerant and sensitive to water-deficit stress observed in this study are in agreement with an earlier study reported for durum wheat (Nouri *et al*. 2011), in which genotypes with high relative water content usually had high stress tolerance under both irrigated and rainfed conditions. Results obtained in this study also show that the decrease of intracellular free water content leads to photosynthetic apparatus damage, which is observed in the reduced levels of chlorophyll content (as indicated in EGA Bellaroi, Tjilkuri and Caparoi). By contrast, small reductions in leaf water potential and relative water content in durum genotypes tolerant to water-deficit stress (as shown in this study with Tamaroi, Yawa and WID802) indicate the maintenance of high turgor potential and adapted osmotic adjustment ability, which is associated with high photosynthetic rate and decreased transpiration rate (Tardieu and Tuberosa 2010; Anjum *et al*. 2011; Tardieu *et al*. 2014). Correlation analysis of leaf water potential and yield components suggests moderate positive associations between the maintenance of plant water potential and yield potential. However, the yield potential of a genotype is complicated by many factors, and yield performance under water-deficit stress is not dependent solely on its level of physiological adaptation.

Conclusion

In water-limiting environments, shortage of soil moisture lowers the water status of the plant, leading to reduced turgor and photosynthetic activity, which ultimately reduces plant growth and yield production. Loss of photosynthetic activity during the reproductive stages of development might lead to decreased pollen viability and thus increased spikelet abortion. Ultimately, this results in reduced grain number, which has a significant impact on the grain yield obtained. The maintenance of high plant water status and maintenance of photosynthetic rate when water-deficit stress occurs at the early stages of reproductive processes are the major physiological attributes of high yield stability in water-deficit-tolerant genotypes under Mediterranean rainfed conditions such as in South Australia. These physiological attributes significantly affect final grain yield and straw yield production. As seen in this study, morphological traits also contribute significantly to yield stability in water-limiting environments. Positive correlations of plant height and fertile tiller number with grain yield and biomass make it possible to evaluate genotypes with high yield stability by using these morphological attributes in rainfed conditions. Significant differences between genotypes tolerant and sensitive to water-deficit stress when investigating the morphological and physiological attributes reported in this study indicate the potential for screening durum wheat genotypes for stress-tolerance improvement in Mediterranean environments.

Acknowledgements

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Chapter 4

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Contribution to the Paper	Designed the experiments, conducted the research, analysed the data and drafted the manuscript.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate to include the publication in the thesis; and

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RESEARCH ARTICLE

Genome-Wide Identification of MicroRNAs in Leaves and the Developing Head of Four Durum Genotypes during Water Deficit Stress

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that play critical roles in plant development and abiotic stress responses. The miRNA transcriptome (miRNAome) under water deficit stress has been investigated in many plant species, but is poorly characterised in durum wheat (*Triticum turgidum* L. ssp. *durum*). Water stress during early reproductive stages can result in significant yield loss in durum wheat and this study describes genotypic differences in the miRNAome between water deficit tolerant and sensitive durum genotypes. Small RNA libraries (96 in total) were constructed from flag leaf and developing head tissues of four durum genotypes, with or without water stress to identify differentially abundant miRNAs. Illumina sequencing detected 110 conserved miRNAs and 159 novel candidate miRNA hairpins with 66 conserved miRNAs and five novel miRNA hairpins differentially abundant under water deficit stress. Ten miRNAs (seven conserved, three novel) were validated through qPCR. Several conserved and novel miRNAs showed unambiguous inverted regulatory profiles between the durum genotypes. Several miRNAs also showed differential abundance between two tissue types regardless of treatment. Predicted mRNA targets (130) of four novel durum miRNAs were characterised using Gene Ontology (GO) which revealed functions common to stress responses and plant development. Negative correlation was observed between several target genes and the corresponding miRNA under water stress. For the first time, we present a comprehensive study of the durum miRNAome under water deficit stress. The identification of differentially abundant miRNAs provides molecular evidence that miRNAs are potential determinants of water stress tolerance in durum wheat. GO analysis of predicted targets contributes to the understanding of genotypic physiological responses leading to stress tolerance capacity. Further functional analysis of specific stress responsive miRNAs and their interaction with targets is ongoing and will assist in developing future durum wheat varieties with enhanced water deficit stress tolerance.

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Introduction

Durum wheat (*Triticum turgidum* L. ssp. *durum*) is the only tetraploid wheat species ($2n = 4x = 28$, genomes AABB) grown commercially throughout the world. Water deficit stress is one of the main abiotic factors that cause durum yield loss in Mediterranean environments. Water deficit stress in early reproductive stages has been shown to adversely affect grain yield and biomass through reduced grain number in durum [1]. Nonetheless, Liu et al. also demonstrated that genotypic variation in morphological and physiological responses exists in durum wheat when grown in water limited conditions [1]. Investigating water deficit stress tolerance mechanisms and genotypic differences within a plant species is an important strategy for understanding the basis of stress response and for selection of genotypes with improved water stress tolerance. The genetic mechanism(s) associated with tolerance against abiotic stresses is not well documented in durum wheat, partly because the full genome sequence is still unavailable. Understanding gene regulatory pathways underlying stress responses may lead to new strategies to enhance stress tolerance in durum wheat.

In plants, small non-coding RNAs of 20–24 nucleotides (nts) have been identified as important regulators of genome integrity, virus and pathogen defence, development and importantly, abiotic stress response pathways [2–4]. Small RNAs are broadly divided into microRNAs (miRNAs) and small interfering RNAs (siRNAs). MicroRNAs are global regulators of gene expression mainly through post-transcriptional repression or translational inhibition [5–7]. The general molecular networks related to their complex biogenesis and silencing have now been widely characterised [8–11]. Plant miRNAs control the expression of their targets by binding to imperfect reverse complementary sequences, resulting in degradation and/or translational repression of the cognate target mRNAs [5,11].

Functional analyses of miRNAs and their targets in plants have demonstrated that miRNAs are associated with diverse biological processes including reproductive development and abiotic stress tolerance [12–14]. A large number of studies with different plant models have revealed the up- or down-regulation of certain responsive miRNAs when subjected to various abiotic stresses such as water deficit, salinity, heat and cold stress (Table 1). Stress-responsive miRNAs have displayed different regulation patterns between species. However, some stress responsive miRNAs might also exhibit different expression patterns when comparing genotypes of the same plant species; as shown in cowpea exposed to drought stress [15], wheat exposed to dehydration stress [16] and maize exposed to salt stress [17]. Such genotype-specific responses of miRNA help explain the genetic basis of the phenotypic and physiological differences between genotypes of the same species under stress conditions [15,18]. Furthermore, miRNAs have been shown to display spatio-temporal patterns specific to certain plant tissues, suggesting the involvement of tissue-specific miRNAs in various developmental processes [16–18]. These tissue-specific patterns have been studied in bread wheat [19,20], but not specifically in durum wheat.

As indicated in Table 1, although numerous miRNAs have been identified in many plant species, including cereals like barley (*Hordeum vulgare*) [35–37], rice (*Oryza sativa*) [38,39], *Brachypodium distachyon* [40,41], and bread wheat (*Triticum aestivum*) [16,19]; only one mature miRNA sequence from *Triticum turgidum* is recorded in the current miRBase v21. A holistic evaluation of cereal miRNA-mediated response mechanisms under stress conditions is far from complete [42], with very little known about miRNAs and their regulatory functions in relation to water deficit stress across multiple durum genotypes.

This study provides insight into miRNA-mediated water deficit stress regulatory pathways, using four Australian durum genotypes with different water deficit sensitivity [1]. Using Illumina sequencing, we identified 110 conserved miRNAs and 159 novel miRNA hairpin

Table 1. Stress responsive microRNAs and their response to different abiotic treatments in various plant species.

miRNA	Water deficit	Salinity	Heat	Cold	References
miR156	Ath↑, Ttu↑, Osa↓, Tae↑↓, Zma↑	Ath↑, Zma↓	Tae↑	—-	[19,21–26]
miR159	Zma↑, Osa↓, Tae↑	Ath↑, Tae↓	Tae↑	Tae↓	[21,22,24,25,27]
miR160	Peu↑	—-	Tae↑	—-	[25,28]
miR162	Zma↑, Peu↑	Zma↑	—-	—-	[17,21,28]
miR166	Zma↑, Ttu↓, Osa↓	Zma↑	Tae↑	—-	[22,23,25,26]
miR167	Ath↑, Zma↑	Ath↑, Zma↓	Tae↑	Osa↓	[17,21,24,29,30]
miR168	Zma↓, Osa↓, Tae↓	Tae↓, Ath↑, Zma↑	Tae↑	Tae↓	[17,21,22,24,25,27]
miR169	Ath↓, Osa↑	Ath↑, Zma↑,	Tae↑	Bdi↑	[17,22,24,31,32]
miR170	Osa↓	Ath↑	—-	—-	[22,24]
miR172	Osa↓, Tae↑	Tae↑	Tae↓	Bdi↑, Tae↑	[22,27,31,32]
miR319	Zma↑, Osa↓	Ath↑	Tae↓		[21,22,24,29]
miR393	Tae↑, Ath↑, Osa↑	Tae↑	Tae↑	Tae↓	[24,27,32,33]
miR395	Zma↓, Osa↑	Zma↑	Tae↑		[17,21,22,29]
miR396	Ath↑, Zma↓, Osa↓, Ttu↓	Ath↑, Zma↓		Ath↑	[17,21–24]
miR397	Osa↓, Tae↓	Tae↓		Bdi↑, Tae↓	[22,27,31,34]
miR398	Zma↑, Ttu↑	Ath↓	Ath↓		[21,23,24,34]
miR399	Zma↓	—-	Tae↑	—-	[21,25]
miR408	Ath↑, Osa↓	—-	—-	Ath↑	[22,24]
miR528	Zma↓, Ttu↓	Zma↑	—-	—-	[17,21,23]
miR529	Osa↓	Tae↓	—-	Tae↑	[22,27]
miR827	Zma↑	—-	Tae↑	—-	[21,25]
miR1029	Tae↑	Tae↓	—-	Tae↑	[27]

Ath, *Arabidopsis thaliana*; Bdi, *Brachypodium distachyon*; Peu, *Populus euphratica*; Ttu, *Triticum turgidum ssp*. *dicoccoides*; Osa, *Oryza sativa*; Zma, *Zea mays*; ↑ = up-regulated; ↓ = down-regulated; —- = not determined

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candidates in durum. Statistical analysis has revealed 66 conserved water deficit stress responsive miRNA as well as a number of conserved tissue- and genotype-specific miRNAs. In addition, 16 conserved and five novel miRNA hairpins showed contrasting regulatory patterns under water deficit stress between stress tolerant and sensitive genotypes. To our knowledge, this is the first report of water deficit stress responsive miRNAs identified through direct small RNA cloning and sequencing in durum wheat. Furthermore, target prediction and Gene Ontology (GO) analysis suggests that miRNA targets function in a broad range of biological processes such as metabolic process, response to stimuli, reproduction and development. Comparisons of miRNA profiles in different genotypes under stress in combination with the investigation of target functions and their gene ontologies is a promising approach in predicting miRNA-mediated stress signalling mechanisms in durum wheat, which may have the potential for improving abiotic stress tolerance in breeding programs [42,43].

Results

Conserved and novel miRNAs in durum discovered using two bioinformatics approaches

To identify conserved and novel miRNAs in durum, 96 sRNA libraries were constructed from flag leaf and head samples from four durum genotypes and sequenced using Illumina high-throughput technology (deposited in NCBI GEO Database, accession number GSE69339).

Approximately 623.4 million reads were obtained from these 96 libraries which represent 16 biological groups (four durum genotypes from each of two tissue types and two water deficit stress treatment groups with six biological replicates in each) (S1 Table). The average number of reads per library was approximately 6.5 million.

For conserved miRNA identification, Approach #1 was developed using CLC Genomics Workbench v7.0 (CLC Bio, Denmark). Approximately 602.1 million reads (that is, 6.3 million per library on average) were obtained after removing low quality sequences, those without inserts, or those with adapter contaminants or lengths outside of the 15–50 nt range. Among the trimmed reads, approximately 301.8 million non-redundant unique small RNA reads were obtained. The most abundant sRNA reads were 21–24 nucleotides (nt), with 24 nt reads being the most common in length (S1 Fig). Unique, mature plant miRNA sequences from nine common monocot and dicot species (*Triticum aestivum*, *Triticum turgidum*, *Brachypodium distachyon*, *Zea mays*, *Oryza sativa*, *Hordeum vulgare*, *Sorghum bicolor*, *Arabidopsis thaliana*, and *Glycine max*) deposited in miRBase were used as references to identify conserved miRNAs in durum wheat allowing a maximum of two mismatches in alignment. Approximately 21.6 million sRNA reads were annotated in 96 libraries, and nearly 2 million annotated tags matched 110 conserved miRNAs in the nine selected plant species (S2 Table).

For novel miRNA identification, a customised bioinformatics approach (Approach #2) was developed. Putative miRNA hairpins were identified using the latest International Wheat Genome Sequencing Consortium's (IWGSC) Chromosomal Survey Sequences (CSS) of bread wheat [44], due to the limited availability of durum wheat sequence. This process resulted in the identification of an initial set of 6,643 loci representing 3,421 non-redundant sequences. Of these non-redundant sequences, 2,710 sequences passed checks by RNAFold and miRcheck, which satisfied *in silico* requirements of the biogenesis pathway of miRNAs in plants. Of these 2,710 candidate miRNA hairpin sequences, 237 matched the expectations for a true miRNA in terms of their read coverage profile (Category A) using three Boolean metrics as described in the Materials and Methods. Of these, 78 contained an exact match to at least one known mature miRNA from miRBase (Table 2), while the remaining 159 putative novel miRNAs had no match to any known mature miRNAs in the miRBase (S3 Table).

Table 2. Summary of putative miRNA hairpins in durum wheat small RNA libraries.

Category	≥95% Strand Bias	≥95% reads in one of the terminal 50bp	≤5% reads in loop region	Number of miRNA hairpins	Number of hairpins with known miRNA	Number of hairpins with putative novel miRNA
A	Y	Y	Y	237	78	159
B	Y	Y	N	96	33	63
C	Y	N	Y	100	55	45
D	Y	N	N	322	72	250
E	N	Y	Y	93	4	89
F	N	Y	N	161	17	144
G	N	N	Y	145	17	128
H	N	N	N	1556	134	1422
Total				2710	410	2300

All putative miRNA hairpin sequences were classified into one of eight categories (A-H, where category A candidates have a read coverage profile matching the expectations for a true miRNA) using 3 Boolean metrics based on the read coverage profile: 1) If ≥ 95% of the reads mapped to one strand of the hairpin; 2) If ≥ 95% of the reads mapped to one of the terminal 50 bp of the hairpin; 3) If ≤ 5% of the reads mapped to the loop region of the hairpin.

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Some conserved durum miRNAs are genotype- or tissue-specific regardless of water-deficit stress (Approach #1)

Differential miRNA expression profiles were observed between the water deficit stress sensitive (EGA Bellaroi and Tjilkuri) and tolerant (Tamaroi and Yawa) genotypes across both treatments. Comparisons were made between Tamaroi versus EGA Bellaroi, and Yawa versus Tjilkuri, separately, based on their breeding history and genetic background. A total of 70 miRNAs were differentially expressed between different durum genotypes (Fig 1). Among these miRNAs, four groups displayed interesting expression patterns between the water deficit stress tolerant and the sensitive genotypes (Table 3): I) miRNAs predominantly expressed in water deficit tolerant genotypes under both treatments (7 miRNAs); II) miRNAs predominantly expressed in the water deficit sensitive genotypes under both treatments (5 miRNAs); III) miRNAs predominantly expressed in the water deficit sensitive genotypes under water deficit stress treatment, but predominantly expressed in the water deficit tolerant genotypes under the control treatment (9 miRNAs); IV) miRNAs predominantly expressed in the water deficit tolerant genotypes under the water deficit stress treatment, but predominantly expressed in the water deficit sensitive genotypes under the control treatment (1 miRNA). For example, in group I, the expression level of Osa-miR5077 was more abundant in Tamaroi compared to EGA Bellaroi in both tissues under both treatments (1.95 fold in control flag leaf libraries, 2.41 fold in water deficit flag leaf libraries, 1.58 fold in control head libraries and 1.60 fold in water deficit head libraries respectively) (Table 3). In group III, Osa-miR5071 was more abundant in Yawa compared to Tjilkuri in the control treatment libraries (1.78 fold in the flag leaf and 2.10 fold in the developing head, respectively); but was more abundant in EGA Bellaroi compared to Tamaroi in the water deficit treatments (1.78 fold in the flag leaf and 1.56 fold in the developing head, respectively) (Table 3).

A comparison between all flag leaf and developing head samples identified miRNAs displaying differential abundance between different tissues, irrespective of genotype and treatment. While a total of 110 conserved miRNAs were identified in all sRNA libraries, 86 miRNAs were differentially abundant between flag leaf tissue and the developing head tissue (Fig 2). A total of nine miRNAs were predominantly expressed in the developing head tissue in all four durum genotypes across both treatments while 37 miRNAs were predominantly expressed in the flag leaf tissue (Table 4). For example, Bdi-miR171d was more abundant (from 2.99 to 9.35 fold greater) in the developing head libraries compared to the flag leaf libraries in the four durum genotypes irrespective of the treatment. In contrast, Tae-miR156 was more abundant (from 4.60 to 8.66 fold greater) in the flag leaf libraries compared to the developing head libraries in the four durum genotypes irrespective of the treatment (Table 4).

Water deficit stress-responsive conserved miRNAs in durum (Approach #1)

Differential expression of conserved miRNAs were found between water deficit stressed and corresponding control libraries in both the flag leaf and developing head tissues of each durum genotype. Using the criteria described in the Materials and Methods, 66 conserved mature miRNAs were determined to be water deficit stress-responsive miRNAs (Fig 3 and S4 Table).

Hierarchical clustering of the water deficit stress-responsive miRNAs illustrated that several miRNAs showed different regulation patterns under water deficit stress between stress tolerant and sensitive genotypes (Fig 3), whereas certain miRNAs showed the same regulation patterns (e.g. Gma-miR408d was up-regulated under stress of all four durum genotypes in the flag leaf tissues). More interestingly, a small number of stress responsive miRNAs showed up-regulation in water deficit stress sensitive genotypes while those same miRNAs were down-regulated

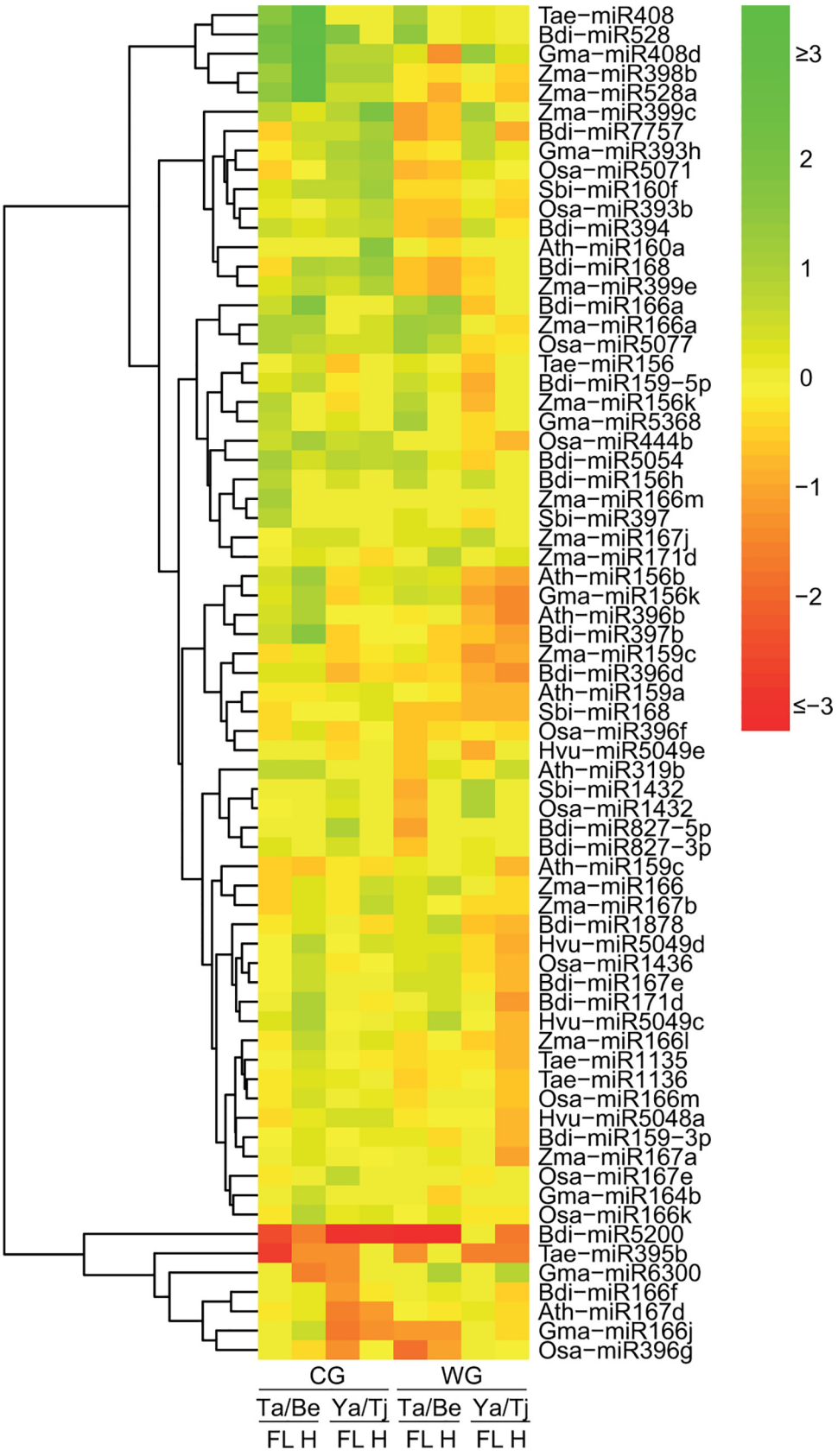
Tae−miR408
Bdi−miR528
Gma−miR408d
Zma−miR398b
Zma−miR528a
Zma−miR399c
Bdi−miR7757
Gma−miR393h
Osa−miR5071
Sbi−miR160f
Osa−miR393b
Bdi−miR394
Ath−miR160a
Bdi−miR168
Zma−miR399e
Bdi−miR166a
Zma−miR166a
Osa−miR5077
Tae−miR156
Bdi−miR159−5p
Zma−miR156k
Gma−miR5368
Osa−miR444b
Bdi−miR5054
Bdi−miR156h
Zma−miR166m
Sbi−miR397
Zma−miR167j
Zma−miR171d
Ath−miR156b
Gma−miR156k
Ath−miR396b
Bdi−miR397b
Zma−miR159c
Bdi−miR396d
Ath−miR159a
Sbi−miR168
Osa−miR396f
Hvu−miR5049e
Ath−miR319b
Sbi−miR1432
Osa−miR1432
Bdi−miR827−5p
Bdi−miR827−3p
Ath−miR159c
Zma−miR166
Zma−miR167b
Bdi−miR1878
Hvu−miR5049d
Osa−miR1436
Bdi−miR167e
Bdi−miR171d
Hvu−miR5049c
Zma−miR166l
Tae−miR1135
Tae−miR1136
Osa−miR166m
Hvu−miR5048a
Bdi−miR159−3p
Zma−miR167a
Osa−miR167e
Gma−miR164b
Osa−miR166k
Bdi−miR5200
Tae−miR395b
Gma−miR6300
Bdi−miR166f
Ath−miR167d
Gma−miR166j
Osa−miR396g
≥3
2
1
0
−1
−2
≤−3
CG
WG
Ta/Be
Ya/Tj
Ta/Be
Ya/Tj
FL H FL H FL H FL H

Fig 1. Heat-map showing differential expression patterns of conserved miRNAs between different genotypes revealed by high-throughput sequencing. The colour scale is based on the log2 value of the fold-change of the water deficit stress tolerant variety (Tamaroi or Yawa) libraries compared to the water deficit stress sensitive variety (EGA Bellaroi or Tjilkuri) libraries. Log2 value = log2 (RPM of miRNA reads in Tamaroi library/RPM of miRNA reads in EGA Bellaroi library) or log2 (RPM of miRNA reads in Yawa library/RPM of miRNA reads in Tjilkuri library). The red colour indicates that the miRNA was more abundant in the water deficit stress sensitive variety; while the green colour indicates that the miRNA was more abundant in the water deficit stress tolerant variety. CG = Control group; WG = Water deficit stress group; FL = Flag leaf samples; H = Head samples; Be = EGA Bellaroi; Ta = Tamaroi; Tj = Tjilkuri; Ya = Yawa; Ath = *Arabidopsis thaliana*; Bdi = *Brachypodium distachyon*; Hvu = *Hordeum vulgare*; Gma = *Glycine max*; Osa = *Oryza sativa*; Sbi = *Sorghum bicolor*; Tae = *Triticum aestivum*; Zma = *Zea mays*.

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in the tolerant genotypes. For example, in the developing head libraries, Bdi-miR7757 was up-regulated in the sensitive genotypes (EGA Bellaroi and Tjilkuri), but was down-regulated in the tolerant genotypes (Tamaroi and Yawa) (Fig 3 and S4 Table). Moreover, some miRNAs responded to water deficit stress only in stress tolerant or sensitive genotypes. In the head libraries, there were 26 miRNAs that were only down-regulated in the stress tolerant genotype Yawa, but not in the stress sensitive genotypes EGA Bellaroi or Tjilkuri (Fig 3). In summary, through further analysing the differentially expressed miRNAs identified through Approach #1, 57 conserved miRNAs were identified as being responsive to water deficit stress, as well as being differentially abundant across different genotypes and tissue types (Fig 4).

Conserved and novel miRNA hairpins showed inverted expression profiles in response to water deficit stress across genotypes (Approach #2)

Using the Limma Bioconductor package [45,46], 23 of the 237 putative miRNA hairpins in Category A were found to have a significant tolerance × treatment interaction term. On manual inspection of the miRNA hairpin read-coverage profiles in Category A, 21 of these 23 miRNA hairpins represent strong candidates as they have good read-coverage signatures (Fig 5). Of these 21 candidates, we determined that 16 perfectly matched at least one known mature miRNA in the miRBase, with some hairpins matching to the same conserved miRNA (Fig 6A). The remaining five novel candidate miRNA hairpins, representing four mature novel miRNAs, do not contain a perfect alignment to any known mature miRNAs (Fig 6B and S2 Fig). For example, miRNA hairpin Ttu pre-miR008 representing Ttu-miR008 was down-regulated in both flag leaf and developing head tissues under water deficit stress in the stress tolerant genotypes (Tamaroi and Yawa), but was up-regulated in the stress sensitive genotypes (EGA Bellaroi and Tjilkuri).

Validation of differentially expressed miRNAs in durum wheat by quantitative real-time PCR (qPCR)

To validate differentially expressed durum miRNAs predicted by high-throughput sequencing, miRNA was quantified using qPCR. Ten selected stress responsive durum miRNA candidates including seven conserved miRNAs (identical to Ath-miR167d, Gma-miR408d, Bdi-miR5054, Osa-miR5071, Bdi-miR5200, Bdi-miR528 and Zma-miR528a) and three novel miRNAs (Ttu-miR007, Ttu-miR038 and Ttu-miR109) were screened using flag leaf and developing head tissues of four durum genotypes simultaneously. Comparative fold changes of expression levels of miRNA are shown in Fig 7. The expression level changes of conserved miRNAs detected by qPCR were compared with those determined by Illumina sequencing (S5 Table). Most miRNAs showed similar trends in their expression profile across Illumina sequencing results and qPCR results. For example, in the Illumina sequencing analysis, Zma-miR528a was determined

Table 3. Genotype-specific durum miRNAs showed four different regulation patterns to water deficit stress.

Name	Resource species in miRBase	Group	CG				WG			
			Ta vs. Be		Ya vs. Tj		Ta vs. Be		Ya vs. Tj	
			FL	H	FL	H	FL	H	FL	H
miR160f	Sbi	I		**1.54**	**1.60**	**2.27**				
miR166a	Zma	I	**1.90**	**1.92**			**2.30**	**2.21**		
miR393h	Gma	I			**2.00**	**2.23**			**1.60**	
miR408	Tae	I	**3.04**	**17.98**			**2.18**			
miR5054	Bdi	I	**2.03**		**1.70**	**1.55**	**1.75**			
miR5077	Osa	I	**1.95**	**1.58**			**2.41**	**1.60**		
miR528	Bdi	I	**4.42**	**9.23**	**3.34**		**2.86**			
miR166j	Gma	II			3.22	2.53	2.25	2.26		
miR395b	Tae	II	6.65	2.51	2.54		2.59		2.96	3.03
miR396d	Bdi	II			1.64				1.88	2.50
miR396g	Osa	II			2.59		3.46	2.04		
miR5200	Bdi	II	5.88	2.88	9.21	7.74	12.94	12.69		3.12
miR156k	Gma	III		**1.95**					2.01	2.81
miR168	Bdi	III		**1.91**	**1.72**	**2.44**	1.59	1.89		
miR319b	Ath	III	**1.53**	**1.55**			1.52			
miR393b	Osa	III				**1.81**	1.56	1.59		
miR398b	Zma	III	**2.42**	**10.11**	**1.95**	**1.97**				
miR399e	Zma	III		**1.60**		**1.87**	1.58	1.89		
miR444b	Osa	III		**2.03**		**1.65**				1.68
miR5071	Osa	III			**1.78**	**2.10**	1.78	1.56		
miR528a	Zma	III	**2.84**	**9.75**				1.88		1.55
miR6300	Gma	IV		2.95	2.48			**1.85**		**1.74**

Fold changes have been determined by comparing the reads per million (RPM) between Tamaroi and EGA Bellaroi, Yawa and Tjilkuri in different treatment groups, and different tissues. Bold fold change values indicate that the miRNA reads were more abundant in the water deficit stress tolerant genotypes (Tamaroi or Yawa), while unbolded fold change values indicate that miRNA reads were more abundant in the water deficit stress sensitive genotypes (EGA Bellaroi or Tjilkuri). Blanks indicate that the fold change is either under 1.5 or the fold change is undetermined due to low abundance in the sequencing libraries. Four groups of miRNAs showed interesting expression patterns between the water deficit stress tolerant/sensitive genotypes: I) miRNAs predominantly expressed in the water deficit tolerant genotypes under both treatments; II) miRNAs predominantly expressed in the water deficit sensitive genotypes under both treatments; III) miRNAs predominantly expressed in the water deficit sensitive genotypes under the water deficit stress treatment but predominantly expressed in the water deficit tolerant genotypes under the control treatment; IV) miRNAs predominantly expressed in the water deficit tolerant genotypes under the water deficit stress treatment but predominantly expressed in the water deficit sensitive genotypes under the control treatment. CG = Control group; WG = Water deficit stress group; FL = Flag leaf libraries; H = Head libraries; Be = EGA Bellaroi; Ta = Tamaroi; Tj = Tjilkuri; Ya = Yawa; Ath = *Arabidopsis thaliana*; Bdi = *Brachypodium distachyon*; Gma = *Glycine max*; Osa = *Oryza sativa*; Sbi = *Sorghum bicolor*; Tae = *Triticum aestivum*; Zma = *Zea mays*.

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to be down-regulated under stress in the head libraries of Tjilkuri, Tamaroi and Yawa (1.2, 5.1, and 2.7 fold reduction), and up-regulated in EGA Bellaroi (3.6 fold increase). When tested by qPCR, the same miRNA was up/down-regulated in the same libraries and varieties (2.6, 4.9, 1.6 fold reduction, and 2.2 fold increase, respectively). While the expression values between the two platforms are not exactly the same, this has been reported previously and is expected based on the two different quantification methods used [47].

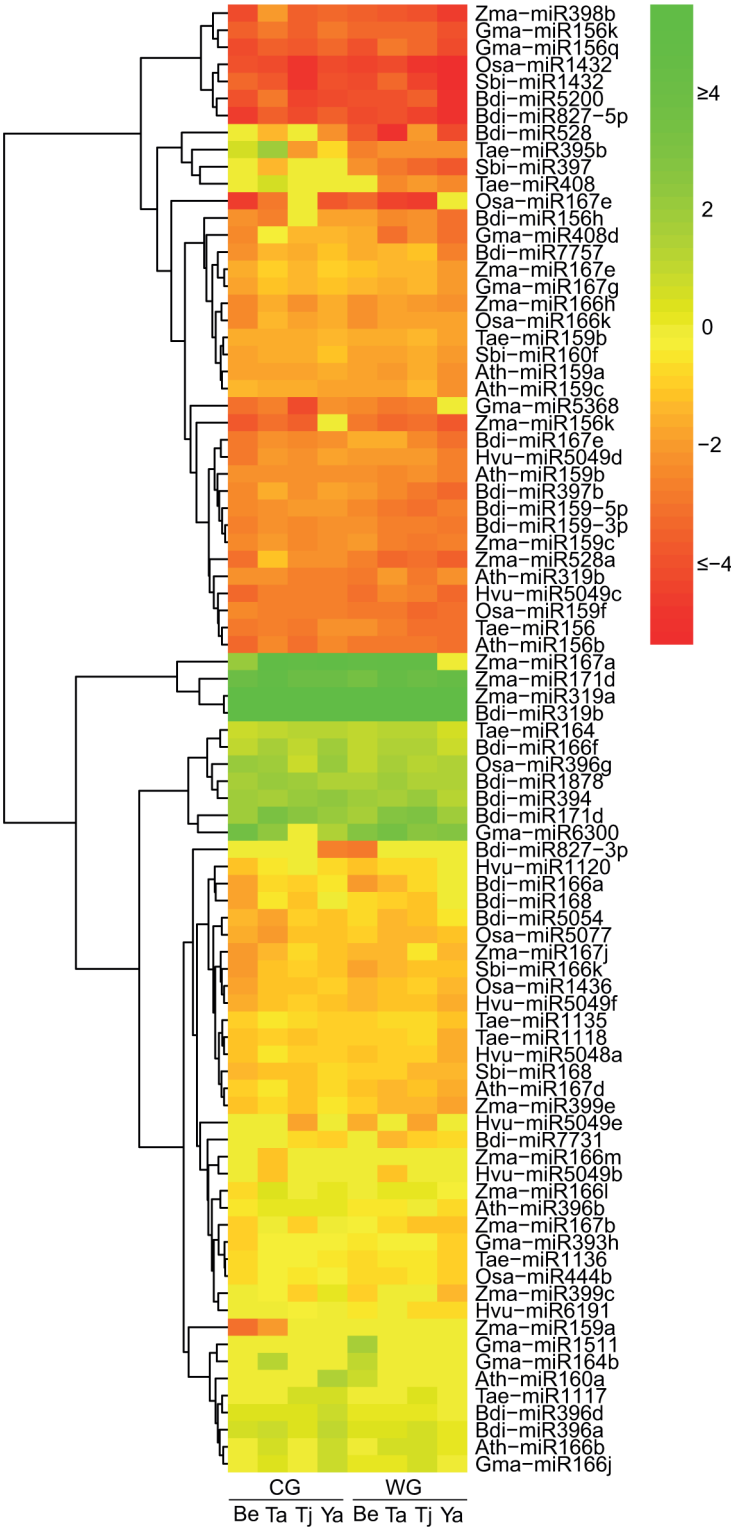

Fig 2. Heat-map showing differential expression patterns of conserved miRNAs between different tissues revealed by high-throughput sequencing. The colour scale is based on the log2 value of the fold-change of the developing head libraries compared to the flag leaf libraries in four durum genotypes under different water treatments. Log2 value = log2 (RPM of miRNA reads in head libraries/RPM of miRNA reads in flag leaf libraries). The red colour indicates that the miRNA was more abundant in the flag leaf libraries; while the green colour indicates that the miRNA was more abundant in the developing head libraries. CG = Control

group; WG = Water deficit stress group; Be = EGA Bellaroi; Ta = Tamaroi; Tj = Tjilkuri; Ya = Yawa; Ath = *Arabidopsis thaliana*; Bdi = *Brachypodium distachyon*; Hvu = *Hordeum vulgare*; Gma = *Glycine max*; Osa = *Oryza sativa*; Sbi = *Sorghum bicolor*; Tae = *Triticum aestivum*; Zma = *Zea mays*.

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Putative targets of novel water deficit stress responsive durum miRNAs, GO analysis and qPCR

To infer the biological functions of the novel water deficit stress responsive miRNAs in durum, putative mRNA target genes were predicted using the psRNAtarget program (http://plantgrn.noble.org/psRNATarget/) with the wheat DFCI gene index (TAGI) version 12 as a reference. A total of 130 targets were identified for four novel stress responsive durum miRNAs (S6 Table). Ttu-miR008 had the highest number of putative target genes (101) while Ttu-miR109 had the lowest (5). On the basis of sequence complementarity between miRNAs and putative target genes, the possible inhibition type between miRNA and their targets was predicted [48,49]. Out of 130 predicted mRNA targets, the inhibition of 109 mRNA targets (83.8%) is caused by cleavage activity, while 21 targets (16.2%) are inhibited through translational repression (S6 Table).

All of the predicted targets were analysed through Gene Ontology (GO) using the Blast2GO server (https://www.blast2go.com/) to further evaluate their putative functions (S6 Table). The BLASTX search obtained the most significant BLAST hits for each target across different species (S3 Fig). According to the ontological definitions of their GO terms, all targets were grouped into three GO categories (S7 Table). At the cellular level (Fig 8A), predicted targets are primarily associated with the nucleus (28.4%), followed by either the mitochondrion or plastid (17.9% each). In evaluating molecular functions, the majority of the targets are potentially involved in either organic or heterocyclic compound binding (16.8% each), ion binding (13.6%), or small molecule binding (10.7%) (Fig 8B). Biologically, nearly half of the targets were classified as being involved in metabolic processes (41.4%) (which includes catabolic, cellular, nitrogen compound, organic substance, primary, and wax metabolic processes) (Fig 8C). The remaining targets were involved in a broad range of biological processes including cellular processes (16.2%), regulation (10.1%), localisation (10.1%), response to stimuli (8.1%), and most significantly, response to stress (5.1%) (Fig 8C). Many of the predicted targets are annotated to be transcription factors, elongation factors, protein phosphatases, and osmotic stress receptors that are associated with multiple stress response processes (S6 Table).

Seven selected targets of Ttu-miR008 were quantified using qPCR (S8 Table). For example, TC438017 (non-specific lipid-transfer protein) and CV779294 (non-specific lipid-transfer protein a-like). In the flag leaf under water stress, TC438017 was up-regulated in the stress tolerant genotypes (4.26 fold in Tamaroi and 2.79 fold in Yawa), whereas it was down-regulated in the stress sensitive genotypes (2.72 fold in EGA Bellaroi and 1.11 fold in Tjilkuri). Similarly, CV779294 was up-regulated in the stress tolerant genotypes (1.34 fold in Tamaroi and 1.40 fold in Yawa), while being down-regulated in the sensitive genotypes (2.37 fold in EGA Bellaroi and 2.41 fold in Tjilkuri). In addition, TC447684 (Glossy 1 protein–GL1) was shown to be up-regulated in the developing head of the stress tolerant genotypes (1.22 fold in Tamaroi and 1.13 fold in Yawa), while being down-regulated in the developing head of the sensitive genotypes (1.17 fold in EGA Bellaroi and 1.52 fold in Tjilkuri). Overall, of the seven targets quantified several were negatively correlated with Ttu-miR008, which was down-regulated in the stress tolerant genotypes but up-regulated in the stress sensitive genotypes (Fig 6B).

Table 4. Durum miRNAs showed tissue-specific expression profiles regardless of water deficit stress.

Name	Resource species in miRBase	H vs. FL							
		CG				WG			
		Be	Ta	Tj	Ya	Be	Ta	Tj	Ya
miR164	Tae	**1.77**	**2.04**	**2.20**	**2.23**	**2.13**	**2.51**	**2.23**	**1.57**
miR166f	Bdi	**2.00**	**3.06**	**1.98**	**3.50**	**2.15**	**2.68**	**2.71**	**1.70**
miR171d	Bdi	**3.66**	**9.35**	**6.06**	**4.48**	**2.99**	**6.81**	**8.08**	**3.43**
miR171d	Zma	**15.80**	**31.10**	**16.08**	**17.38**	**10.62**	**23.51**	**15.62**	**23.46**
miR1878	Bdi	**3.24**	**4.40**	**3.45**	**2.75**	**2.89**	**3.81**	**2.94**	**2.61**
miR319a	Zma	**38.68**	**60.19**	**51.90**	**58.11**	**61.28**	**57.17**	**41.15**	**54.74**
miR319b	Bdi	**86.21**	**106.61**	**90.62**	**97.54**	**111.48**	**102.45**	**63.82**	**71.59**
miR394	Bdi	**3.96**	**3.36**	**4.60**	**4.99**	**3.86**	**3.36**	**4.33**	**2.48**
miR396g	Osa	**4.27**	**3.98**	**1.73**	**4.19**	**1.95**	**3.30**	**2.29**	**2.64**
miR1118	Tae	2.40	1.82	2.18	1.90	1.95	2.04	1.57	2.90
miR1432	Osa	15.95	18.01	27.73	18.03	23.05	17.16	28.02	102.93
miR1432	Sbi	10.47	13.31	27.77	15.61	19.83	9.93	20.66	76.54
miR1436	Osa	3.27	2.09	2.15	1.87	2.60	2.37	1.94	2.64
miR156	Tae	7.89	5.98	7.43	4.60	5.09	6.08	8.34	8.66
miR156b	Ath	9.86	5.49	8.29	5.16	7.06	7.55	6.98	8.90
miR156k	Gma	12.39	7.80	11.52	7.19	9.61	9.91	10.84	15.12
miR156q	Gma	17.46	11.48	13.38	10.47	15.01	7.87	10.07	19.20
miR159-3p	Bdi	5.96	4.59	5.64	4.72	4.65	6.21	6.83	7.23
miR159-5p	Bdi	5.81	4.42	4.31	3.95	5.31	7.18	8.72	6.90
miR159a	Ath	3.65	3.74	3.51	3.17	3.58	3.91	3.05	5.01
miR159b	Ath	4.68	4.59	4.56	4.43	4.65	5.88	4.44	6.16
miR159b	Tae	3.20	3.07	2.82	2.60	3.00	3.21	2.63	3.47
miR159c	Ath	2.65	2.86	2.97	3.27	3.48	3.78	2.44	4.51
miR159c	Zma	5.85	4.34	5.45	4.58	4.03	5.96	7.11	6.06
miR159f	Osa	5.53	6.59	6.36	6.51	7.92	7.56	10.04	8.70
miR160f	Sbi	1.77	2.04	2.20	2.23	2.13	2.51	2.23	1.57
miR166h	Zma	5.39	2.95	5.02	2.82	4.45	3.36	3.97	5.10
miR166k	Sbi	3.86	2.12	2.01	2.17	3.31	2.60	2.26	2.23
miR166k	Osa	5.58	2.67	3.43	3.13	4.93	3.62	3.60	3.62
miR167d	Ath	2.05	1.52	2.15	1.63	2.29	2.62	2.23	2.99
miR167e	Bdi	7.97	4.89	5.28	4.85	3.15	3.25	5.49	8.05
miR167e	Zma	3.09	2.02	2.53	1.92	2.25	2.64	2.73	4.02
miR167g	Gma	3.35	2.41	2.47	2.12	2.76	2.92	2.75	3.98
miR168	Sbi	2.53	2.14	2.34	1.65	2.01	1.95	2.46	2.51
miR319b	Ath	4.66	4.61	6.38	6.53	7.90	4.25	7.84	4.81
miR397b	Bdi	5.57	2.82	4.49	3.34	4.10	5.51	7.14	9.77
miR398b	Zma	18.22	4.36	10.95	10.83	11.54	13.51	16.78	24.72
miR399e	Zma	2.31	1.72	2.20	1.52	2.03	2.43	2.54	3.61
miR5049c	Hvu	10.42	6.64	6.85	6.13	9.31	5.72	6.42	10.15
miR5049d	Hvu	7.61	4.05	4.94	3.43	3.95	4.00	4.23	6.36
miR5049f	Hvu	3.09	2.31	1.88	2.16	2.63	2.22	2.15	3.24
miR5077	Osa	3.11	3.82	2.18	2.21	1.80	2.72	2.44	2.23
miR5200	Bdi	16.15	7.91	21.11	17.74	15.67	15.37	11.86	36.72
miR528a	Zma	8.10	2.36	4.95	5.05	6.43	9.92	9.19	12.06

(*Continued*)

Table 4. (*Continued*)

Name	Resource species in miRBase	H vs. FL							
		CG				WG			
		Be	Ta	Tj	Ya	Be	Ta	Tj	Ya
miR7757	Bdi	5.02	2.49	3.01	2.11	3.21	2.44	2.23	6.69
miR827-5p	Bdi	23.90	11.91	18.34	12.09	18.30	16.37	22.78	48.05

Fold changes have been determined by comparing the RPM between flag leaf libraries and head libraries in four durum wheat genotypes under different water treatments. Bold fold change values indicate that the miRNA reads were more abundant in the head libraries (nine miRNAs), while unbolded fold change values indicate that the miRNA reads were more abundant in flag leaf libraries (37 miRNAs). CG = Control group; WG = Water deficit stress group; FL = Flag leaf libraries; H = Head libraries; Be = EGA Bellaroi; Ta = Tamaroi; Tj = Tjilkuri; Ya = Yawa; Ath = *Arabidopsis thaliana*; Bdi = *Brachypodium distachyon*; Gma = *Glycine max*; Osa = *Oryza sativa*; Sbi = *Sorghum bicolor*; Tae = *Triticum aestivum*; Zma = *Zea mays*.

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Discussion

The miRNAome in durum wheat under water deficit stress

Water deficit is a major abiotic stress that limits the production of many crops in rain-fed environments. Plant responses to water deficit stress are regulated by complex genetic and epigenetic networks. Interactions between miRNAs and their target mRNAs through sequence-specific binding offer an inheritable and accurate regulation pathway for plants to respond to environmental stimuli at both the translational and post-transcriptional level. To date, extensive efforts have been made to discover water deficit stress-associated miRNAs in many plants including Arabidopsis [24], rice [22], maize [50], soybean [51], barley [52] and bread wheat [16,53]. However, there has rarely been any study on water deficit-stress responsive miRNAs in *Triticum turgidum*, with only the ssp. *dicoccoides* being investigated but under shock drought conditions [23]. As an important cereal, mostly grown in rain-fed Mediterranean environments under stressful and variable conditions, durum wheat offers an attractive alternative to studying the much more complex bread wheat genome. With climate change models predicting increased rising temperatures and decreased rainfall, understanding the water deficit stress response pathway(s) in durum wheat has become an important research objective for breeding programs.

Using deep sequencing of small RNA libraries in this study, we discovered significant changes that occur with the miRNAome in four durum genotypes under water deficit stress and across two tissue types. Illumina sequencing yielded approximately 623 million reads which were subsequently trimmed and processed to remove inherent redundancy, obtaining a total of 301 million unique sRNA sequences. The highest proportion of the sequenced RNAs was 24 nt in length, which is in agreement with previous studies where 24 nt sRNA fragments constituted the majority of small RNA populations, thereby implicating the function of Dicer proteins during the formation of miRNAs [25,29,54]. Since durum wheat ($2n = 4x = 28$, genomes AABB) is an ancestral source of the A and B genomes of bread wheat ($2n = 6x = 42$, genomes AABBDD) and only a partial genome sequence for *Triticum turgidum* ssp. *durum* is available, the International Wheat Genome Sequencing Consortium's (IWGSC) Chromosomal Survey Sequences (CSS) of bread wheat was used to identify novel putative miRNA hairpins in durum sRNA libraries [44]. From the 110 conserved miRNAs and 159 novel miRNA hairpins identified, 66 conserved miRNAs and four novel miRNAs were water deficit stress responsive. Further experimental validation including Poly (A)-qPCR and miRNA* examination will assist in confirming novel durum miRNA hairpins and their precise excision of the miRNA/miRNA* duplex [20,55]. In this study, ten representative stress responsive miRNAs (seven conserved

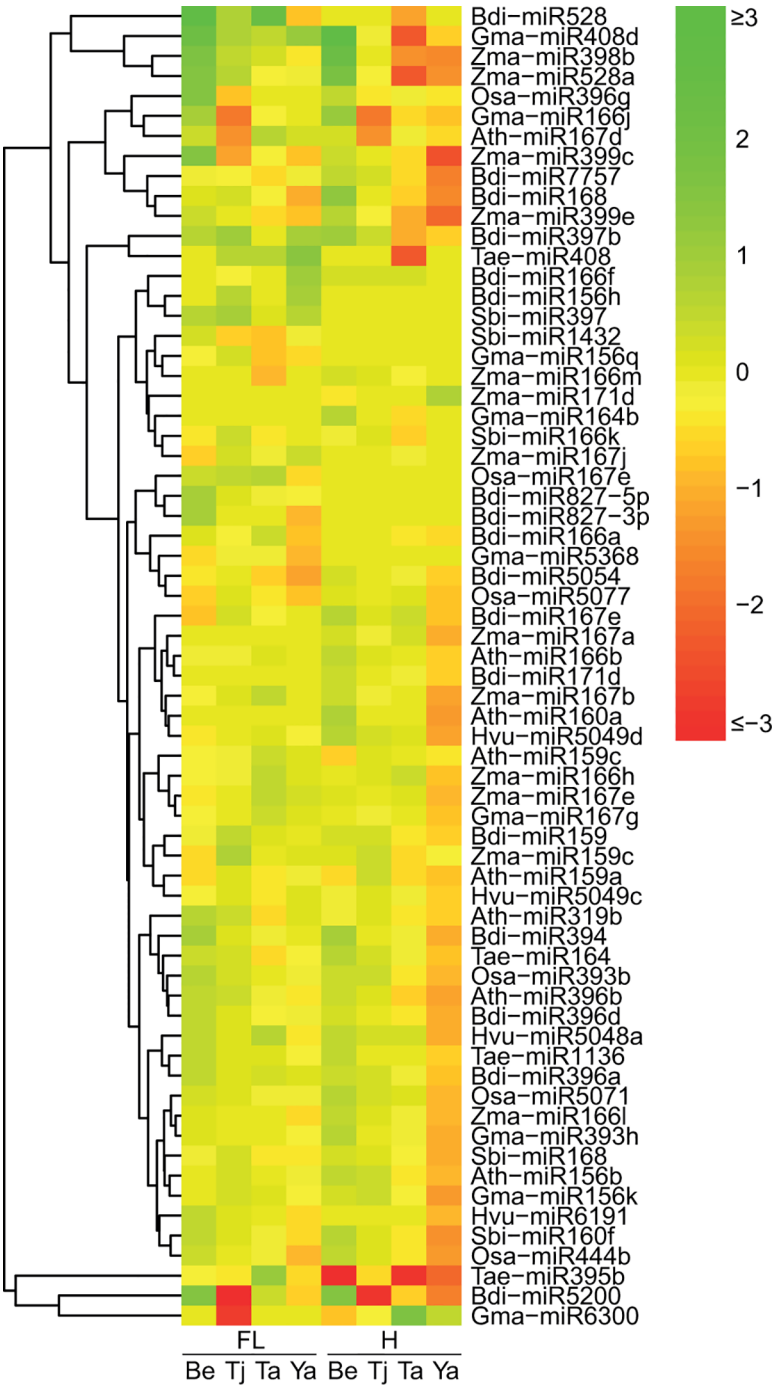

Fig 3. Heat-map showing expression patterns of water deficit stress responsive conserved miRNAs revealed by high-throughput sequencing. The colour scale is based on the log2 value of the fold-change of the water deficit stress treatment libraries compared to the control treatment libraries in four durum genotypes. Log2 value = log2 (RPM of miRNA reads in water deficit stress libraries/RPM of miRNA reads in to control libraries). The red colour indicates that the miRNA was more abundant in the control libraries; while the green colour indicates that the miRNA was more abundant in the water deficit treatment libraries.
CG = Control group; WG = Water deficit stress group; FL = Flag leaf samples; H = Head samples; Be = EGA Bellaroi; Ta = Tamaroi; Tj = Tjilkuri; Ya = Yawa; Ath = *Arabidopsis thaliana*; Bdi = *Brachypodium distachyon*; Hvu = *Hordeum vulgare*; Gma = *Glycine max*; Osa = *Oryza sativa*; Sbi = *Sorghum bicolor*; Tae = *Triticum aestivum*; Zma = *Zea mays*.

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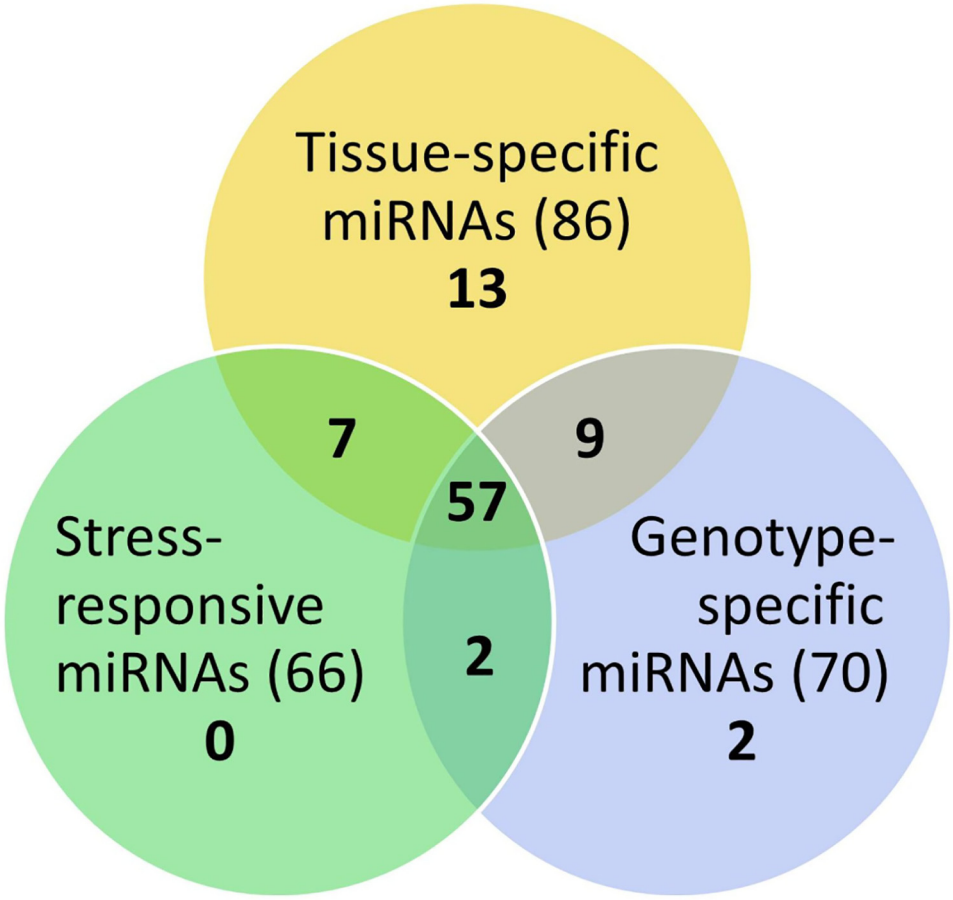

Fig 4. Venn diagram of all differentially expressed conserved microRNAs identified through Approach #1. The number of microRNAs that were differentially abundant in each category is indicated. A total of 57 conserved miRNAs were identified as being responsive to water deficit stress, as well as being differentially abundant across different genotypes and tissue types.

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and three novel) were validated by Poly (A)-qPCR. Poly (A)-qPCR has been shown in bread wheat to provide more accurate and consistent quantification of miRNA expression than stem-loop qPCR [56].

Among water deficit stress responsive miRNAs identified in this study, some miRNAs have been found to be associated with abiotic stress response in previous studies; including miR156, miR159, miR167, miR319, miR393, miR398, and miR408. The expression patterns of some of these water deficit stress responsive miRNA were similar to results previously reported. For example, miR159 was up-regulated 1.75 times under water deficit stress in Tjilkuri. Similarly in maize, the expression level of miR159 was significantly increased during drought stress [21]. The up-regulation of miR162, miR167, miR393 under water deficit stress has been commonly observed in different plants (Table 1), indicating that some miRNA stress-responsive pathways are more than likely to be conserved across different plant species including durum wheat. In contrast, some conserved miRNAs, as well as novel durum miRNAs, were found to be water deficit stress responsive for the first time, including miR1136, miR1432, miR5048, miR5054, miR5071, miR5200 and miR6300. Their regulation pattern indicates that these miRNAs are possibly involved in species-specific response pathways.

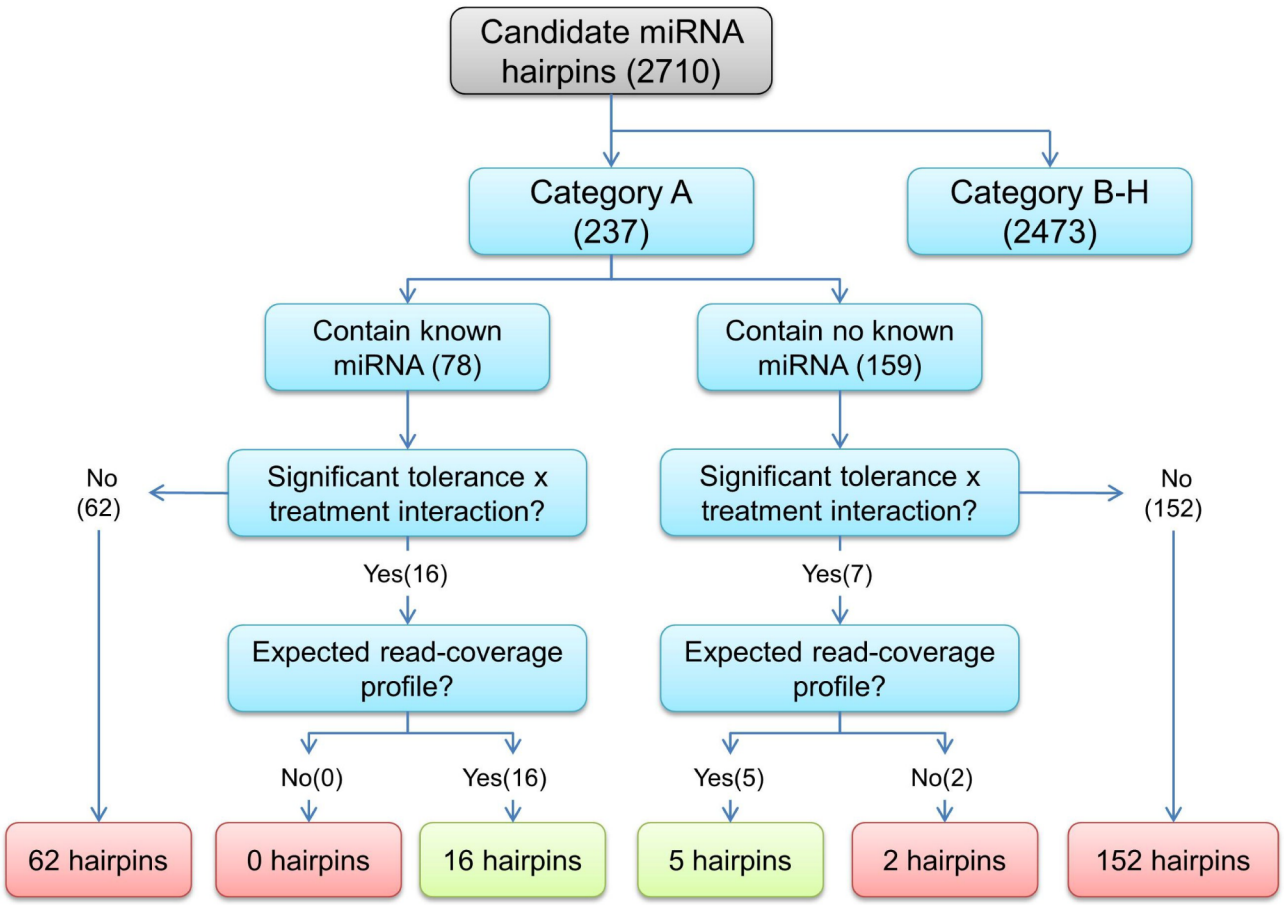

Fig 5. A schematic representation displaying the breakdown of water deficit stress responsive miRNA hairpins identified. A total of 16 hairpins have a significant tolerance × treatment interaction term and contain at least one perfect alignment to a known mature miRNA, while five hairpins have a significant tolerance × treatment interaction term but do not contain a perfect alignment to any known mature miRNAs.

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Most interestingly, the expression profiles of 16 conserved and five novel miRNA hairpins showed inverted regulatory patterns between water deficit stress tolerant and sensitive genotypes, suggesting the regulatory roles of miRNAs in some stress response pathways are genotype-specific (Fig 6). The four durum wheat genotypes used in this study have different levels of water deficit tolerance, which is reflected through their genotypic physiological responses [1]. The distinct genotype differences in miRNA expression profiles could lead to inverted regulation of their functional target genes, which might activate different physiological responses between genotypes [16]. In a recent study of dehydration associated miRNA in wheat, contrasting expression patterns of 13 conserved miRNA (including Tae-miR160a, Tae-miR166h, Tae-miR172a, and Tae-miR393) were also observed between stress tolerant and sensitive genotypes [16]. In the current study, several conserved miRNAs were found to be predominantly expressed in specific genotypes, with or without water deficit stress treatments. For example, miR5200 was consistently more abundant in the water deficit stress sensitive genotypes (EGA Bellaroi and Tjilkuri) than the stress tolerant genotypes (Tamaroi and Yawa) in both the control and water deficit stress libraries. Based on the prediction and further analysis of miRNA targets, we can infer that different capacities for water deficit stress tolerance between durum wheat genotypes may arise from the differential physiological regulation triggered by target genes, which are regulated by genotypic stress responsive miRNAs.

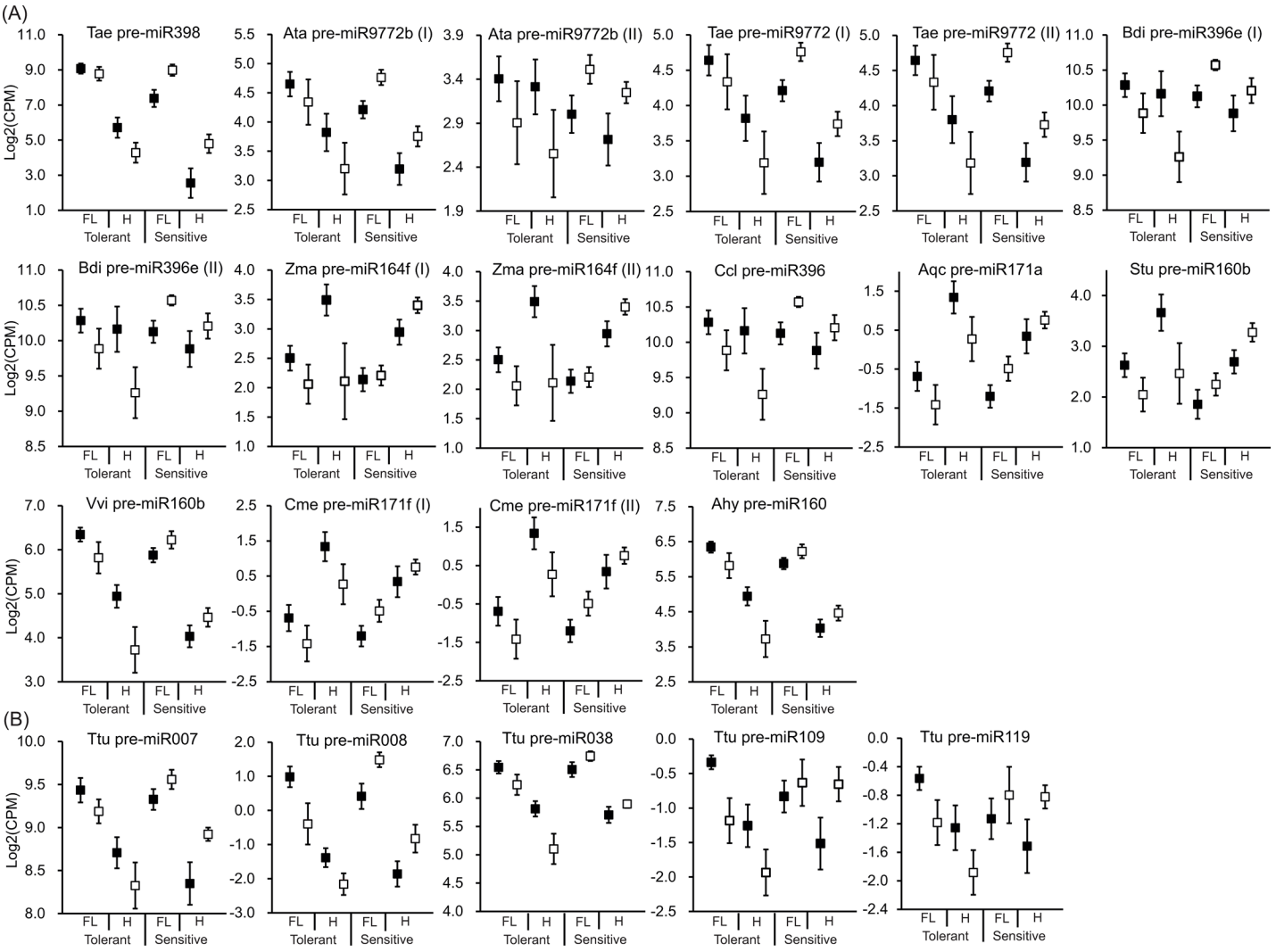

Fig 6. Expression profiles of stress responsive miRNA hairpins showing inverted regulatory patterns between stress tolerant/sensitive genotypes. In (A) 16 conserved miRNA hairpins representing 11 conserved miRNAs are shown, while in (B) five novel miRNA hairpins representing four conserved miRNAs are displayed. The log2 value of normalised reads for each miRNA hairpin is represented as counts per million (CPM). ■ = Control group; □ = Water deficit stress group. Tae = *Triticum aestivum*; Ata = *Aegilops tauschii*; Bdi = *Brachypodium distachyon*; Zma = *Zea mays*; Ccl = *Citrus clementina*; Aqc = *Aquilegia coerulea*; Stu = *Solanum tuberosum*; Vvi = *Vitis vinifera*; Cme = *Cucumis melo*; Aty = *Arachis hypogaea*; Tolerant = Stress tolerant genotypes (Tamaroi and Yawa); Sensitive = Stress sensitive genotypes (EGA Bellaroi and Tjilkuri); I and II denotes two different hairpins representing the same conserved miRNA.

doi:10.1371/journal.pone.0142799.g006

Regulation of miRNA and their targets may contribute to genotypic variation in stress tolerance capacity in different durum genotypes

In the present study, *in silico* target gene predictions and GO analysis were carried out for four novel water deficit stress responsive miRNAs. This bioinformatics strategy has been applied previously in bread wheat to successfully predict and construct possible miRNA/mRNA target stress regulatory pathways, which were further experimentally validated [16,19,53,57–59]. A total of 130 target genes for four novel durum miRNAs were predicted to encode proteins of diverse functions. GO analysis indicated that these targets are involved in a broad range of biological processes and varied physiological responses in durum wheat, such as biosynthetic activity, binding activities with proteins and nucleic acids, protein transport, abscisic acid

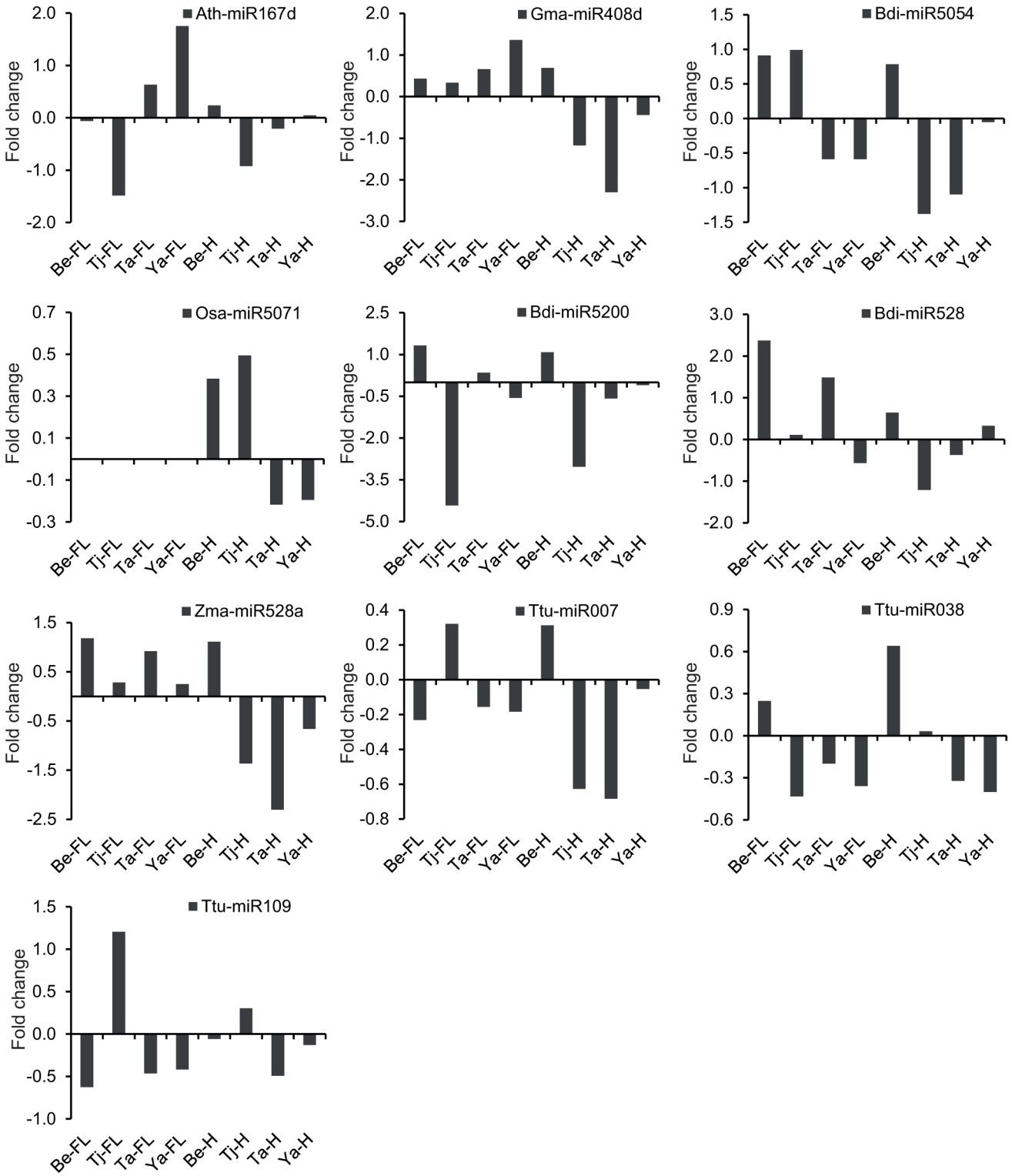

Fig 7. Expression analysis of stress responsive miRNA candidates by qPCR in four durum wheat genotypes. GAPDH was used as an endogenous control. The fold change is shown as a log2 value of miRNA expression in the water deficit libraries/miRNA expression in the control libraries. FL = Flag leaf samples; H = Head samples; Be = EGA Bellaroi; Ta = Tamaroi; Tj = Tjilkuri; Ya = Yawa; Ath = *Arabidopsis thaliana*; Bdi = *Brachypodium distachyon*; Gma = *Glycine max*; Osa = *Oryza sativa*; Ttu = *Triticum turgidum*; Zma = *Zea mays*.

doi:10.1371/journal.pone.0142799.g007

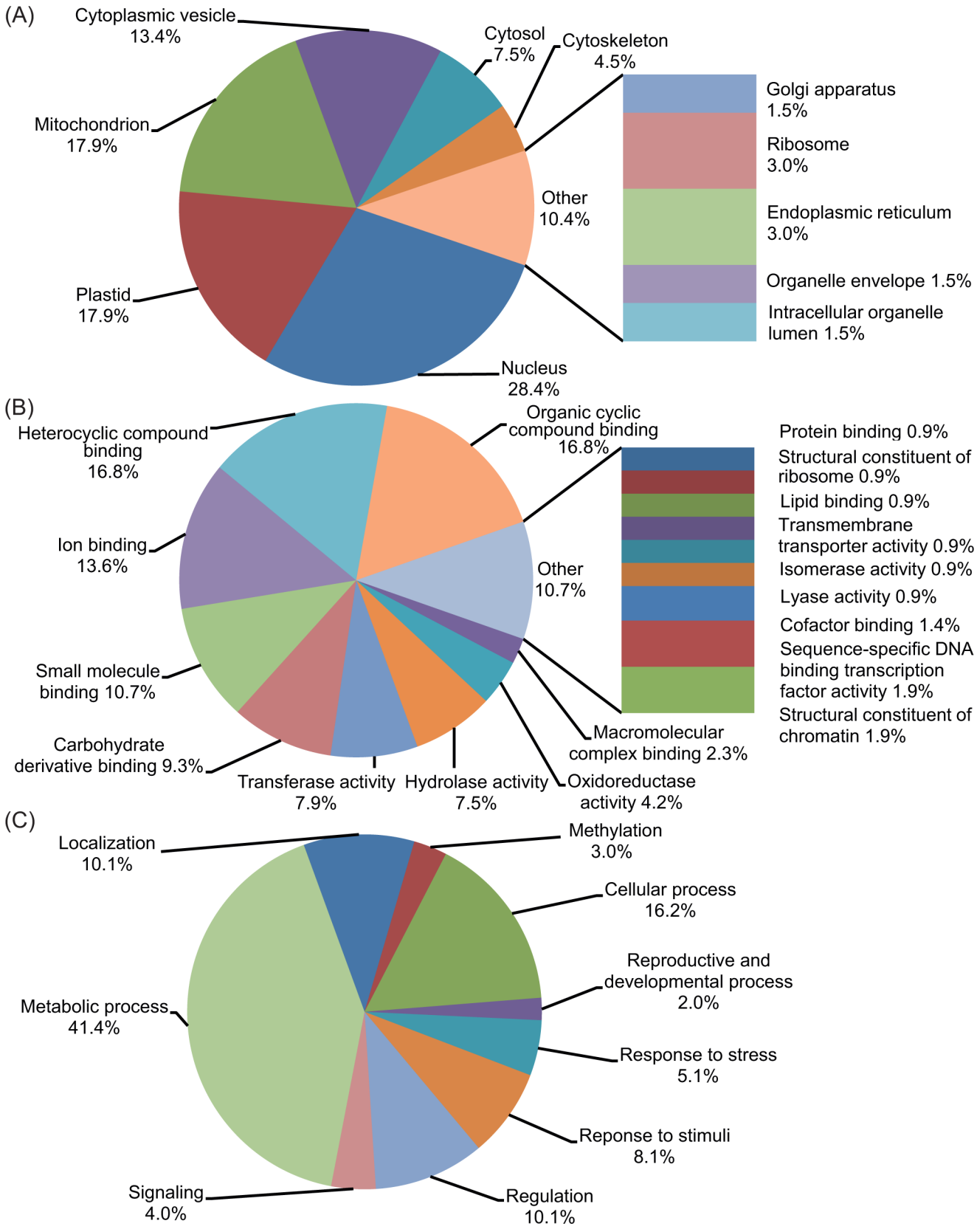
(A)
Cytoplasmic vesicle 13.4%
Cytosol 7.5%
Cytoskeleton 4.5%
Mitochondrion 17.9%
Other 10.4%
Plastid 17.9%
Nucleus 28.4%
Golgi apparatus 1.5%
Ribosome 3.0%
Endoplasmic reticulum 3.0%
Organelle envelope 1.5%
Intracellular organelle lumen 1.5%
(B)
Heterocyclic compound binding 16.8%
Organic cyclic compound binding 16.8%
Ion binding 13.6%
Other 10.7%
Small molecule binding 10.7%
Carbohydrate derivative binding 9.3%
Transferase activity 7.9%
Hydrolase activity 7.5%
Oxidoreductase activity 4.2%
Macromolecular complex binding 2.3%
Protein binding 0.9%
Structural constituent of ribosome 0.9%
Lipid binding 0.9%
Transmembrane transporter activity 0.9%
Isomerase activity 0.9%
Lyase activity 0.9%
Cofactor binding 1.4%
Sequence-specific DNA binding transcription factor activity 1.9%
Structural constituent of chromatin 1.9%
(C)
Localization 10.1%
Methylation 3.0%
Cellular process 16.2%
Reproductive and developmental process 2.0%
Metabolic process 41.4%
Response to stress 5.1%
Reponse to stimuli 8.1%
Signaling 4.0%
Regulation 10.1%

Fig 8. Categorisation of predicted targets of four novel stress responsive miRNAs using Gene Ontology (GO) terms. Pie charts representing different GO categories are based on the number of target sequences enriched in each GO term. GO terms at level 8 are used for (A) cellular component categorisation. GO terms at level 3 are used in categorisation for (B) Molecular function, and (C) Biological processes. The percentage of each GO term is based on the number of targets enriched for that term relative to the total number of targets in each category. The GO level represents the position of a GO term in the GO hierarchy. The level of a GO term is the number of GO terms between that term and the Root Term of the Ontology.

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(ABA) metabolic processes, photosynthetic activity and leaf senescence. Significantly, stress responsive expression of seven predicted target genes were validated by qPCR. The negative correlation of several targets with their corresponding miRNA implies the involvement of miRNA-mRNA target regulation in stress response pathways in durum.

A significant number of targets are predicted to possess nucleic acid binding activities and encode transcription factors involved in signalling and defence, which contribute to stress tolerance in different durum genotypes. For example, auxin response factor (ARF) 18-like is a target of Ttu-miR008. ARFs bind to auxin response elements to usually negatively regulate expression of auxin-inducible genes such as *GH3* (Gretchen Hagen3), *Aux*/*IAA* (auxin/indole-3-acetic acid) and *SAUR* (small auxin-up RNA) [60]. Several auxin-responsive genes have been identified to respond to various abiotic stress conditions such as drought, salinity and cold in Arabidopsis, rice and sorghum, indicating the cross-talk between auxin signalling and abiotic stress responses [61–63]. In durum, Ttu-miR008 is down-regulated under stress in the tolerant genotypes suggesting that ARF18-like protein increases thereby repressing auxin-inducible genes enhancing auxin signalling. This might affect processes which require a lower auxin:cytokinin ratio, such as lateral root development [64]. In maize and wheat, the development of lateral roots in the stress tolerant genotype is enhanced from the accumulation of auxin-responsive factors [16,17]. However, the role of miRNA and ARF in lateral root development in durum needs to be confirmed with further experimental validation.

Other targets also contribute to water stress tolerance in durum as signalling factors including protein kinases and protein phosphatases. For example, a target of Ttu-miR008 (TC451175) was annotated as a probable protein phosphatase 2C (PP2C). Studies in Arabidopsis and rice have shown that PP2C genes were induced by diverse environmental stimuli and acted as positive regulators in ABA-mediated signalling pathways well known to be involved in stress responses [65,66].

However, there are also other targets of Ttu-miR008 which could contribute to water deficit stress tolerance in different ways such as maintaining osmotic pressure of the plant or homeostasis of the cell. For example, the target CV769573 identified in this study as an ABA 8'-hydroxylase, is a key enzyme in ABA degradation [67]. ABA is crucial for various stress responses, including regulation of stress-responsive genes, stomatal closure, and metabolic changes [68]. ABA is rapidly increased in response to environmental stress [67], suggesting a role for removing ABA 8'-hydroxylation to ensure increased ABA levels. Equally rapid elimination of stress induced ABA when stresses are relieved is essential [69]. Indeed, dehydration stress can cause steady increases in ABA degradation in Arabidopsis over time [70]. Although requiring confirmation, ABA 8'-hydroxylase may therefore decrease to a lesser extent in tolerant genotypes suggesting they have a lower ABA requirement during water deficit stress.

Also identified and quantified in this study was the Glossy 1 (GL1) protein, which is yet another target of Ttu-miR008 (TC447684). GL1 functions in the biosynthesis pathway of cuticular wax, which provides protection against environmental stress. In rice, Os*GL1* over-expression plants showed increased cuticular wax accumulation on the leaf surface and were more tolerant to drought stress at reproductive stages compared to the wild type [71]. The inhibition of GL1 is reduced through the down-regulation of stress responsive miRNA, leading to enhanced wax production, thus preventing water loss. This helps to explain the genotypic

difference in the reduction of relative water content in leaves, in response to water deficit stress between stress tolerant and sensitive durum genotypes [1].

Two other quantified functional targets, TC438017 (non-specific lipid-transfer protein) and CV779294 (non-specific lipid-transfer protein a-like), examined by qPCR may also assist to explain the genotypic difference in maintaining osmotic pressure. Lipid transfer proteins (LTPs) help to repair stress-induced damage in membranes or alter the lipid composition of membranes. In pepper, the accumulation of LTP transcripts induced by environmental stresses is associated with cuticle formation, which contributes to the avoidance/tolerance of low tissue water potential and water content [72,73]. In this study, TC438017 and CV779294 were negatively correlated with their corresponding miRNA showing genotypic expression patterns in response to water deficit. The up-regulated accumulation of LTPs observed only in stress tolerant durum genotypes helps to explain the genotypic differences in the maintenance of leaf water potential and relative water content [1], suggesting the participation of miRNA/target interaction in genotypic physiological response pathways in durum. Experimental examination of these miRNA-regulated targets also helps demonstrate the validity of prediction analysis using bioinformatics.

Conclusion

The present study provides a comparative description of the miRNAome in durum wheat between water deficit tolerant and sensitive genotypes in response to water deficit stress, suggesting that there are multiple miRNA regulation patterns which might contribute to, and partly explain, the distinct water deficit stress sensitivities between different durum genotypes. The first comprehensive durum small RNA dataset generated provides a good foundation for future characterisation of the molecular mechanisms underlying water deficit stress tolerance in durum. This was achieved through Illumina sequencing, which enabled profiling of the miRNAome in water deficit stress tolerant and sensitive durum wheat genotypes across different tissues and treatments. We have identified 110 conserved miRNAs and 159 novel miRNA hairpins in durum wheat, including 66 conserved miRNAs and five novel miRNA hairpins (representing four novel miRNAs) that are water deficit stress responsive. A total of 16 conserved miRNA hairpins (representing 11 conserved miRNAs) and five novel miRNA hairpins (representing four novel miRNAs) showed distinct down-regulation profiles in the water deficit stress tolerant genotypes while the same miRNAs were up-regulated in sensitive genotypes. This demonstrates that regulation patterns of the same miRNAs may vary extensively across genotypes of the same species, in response to environmental stimuli. Target prediction and GO analysis of four novel genotype-specific regulated miRNAs provide evidence for the potential involvement of miRNAs in a broad range of biological processes, including stress response pathways. Several potentially valuable target genes have been identified and are now undergoing further experimental validation, which will be reported elsewhere.

Materials and Methods

Plant material and growth conditions

Four durum wheat genotypes (EGA Bellaroi, Tamaroi, Tjilkuri and Yawa) were used in this study. Seeds were obtained from Durum Breeding Australia's (DBA) southern node breeding program (The University of Adelaide). Tamaroi and Yawa are water deficit stress tolerant genotypes; while EGA Bellaroi and Tjilkuri are water deficit stress sensitive genotypes [1]. Plants were grown at 22°C/12°C day/night temperature with a 12 h photoperiod with watering to field capacity (12% soil water content (SWC)) from germination to booting stage when the

water limiting stress treatment was imposed for 15 d (6% SWC or 50% field capacity; water deficit stress group, WG) or field capacity maintained (control, CG), as per Liu et al. [1].

Sampling and total RNA extraction

After 15 d of water deficit stress, the flag leaf and developing head were collected with sterile razor blades and frozen immediately in liquid nitrogen. Frozen tissues were ground to a fine powder in liquid nitrogen using a sterile mortar and pestle, pre-chilled to -80°C. Total RNA was isolated using the TriPure isolation reagent kit (Roche Diagnostics, Australia) and treated with RQ1 RNase-Free DNase I (Promega, Australia) following the manufacturer's instructions. The concentration and quality of extracted RNA samples were measured by spectrophotometric analysis at 260 nm and 280 nm using a NanoDrop Lite spectrophotometer (Thermo Scientific, USA). RNA integrity was assessed by agarose gel electrophoresis. A total of 96 RNA samples (4 durum genotypes × 2 tissue types × 2 treatment groups × 6 biological replicates = 96) were extracted and stored at -80°C for downstream applications.

Small RNA library construction and deep sequencing

For small RNA library construction, 5 μg of total RNA was size-fractionated on a 15% denaturing TBE urea polyacrylamide gel and small RNAs (15 to 40 nt) were excised using an NEB miRNA marker (New England Biolabs, UK) as a guide. Small RNAs was eluted in 0.3 M NaCl by rotating the tube overnight at 4°C. Eluted RNA was passed through a Spin-X column and then precipitated using glycoblue (Ambion, USA) and isopropanol. The sRNA pellets were washed and air-dried at room temperature, then re-suspended in DEPC-treated water [74]. A total of 96 small RNA libraries were constructed from flag leaf and developing head of durum wheat plants that had been treated or not treated with water deficit stress (4 durum genotypes × 2 tissue types × 2 treatment groups × 6 biological replicates = 96) using NEB Next® Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, UK) following the manufacturer's instructions. For each flag leaf sRNA library and head sRNA library, a unique index primer was used for multiplexing purposes using the NEBNext® Index Primer Set (New England Biolabs, UK). The final cDNA product was purified using Pippin Prep™ System (Sage Science, USA). Prior to sequencing, quality and quantity of the amplified small RNA cDNA libraries was evaluated on an Agilent 2100 Bioanalyzer system (Agilent Technologies, USA) and Qubit fluorometer (Invitrogen, USA). All 96 small RNA libraries were sequenced using Illumina sequencing technology on a HiSeq2500 machine after cluster generation. All sequencing reads were submitted to the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/), and are accessible under the accession number GSE69339.

Identification of conserved miRNAs (Approach #1)

In this study, Approach #1 was developed to identify conserved miRNAs in durum wheat using CLC Genomics Workbench v7.0 (CLC Bio, Denmark). Briefly, raw sequencing reads were first processed by trimming adaptor sequences and removing low-quality reads. Sequences shorter than 15 nt and larger than 50 nt were excluded from further analysis. Trimmed reads were generated for each small RNA library and then annotated to determine the presence of known plant miRNAs. Durum small RNA sequences were aligned with known miRNAs in miRBase using CLC Genomics Workbench v7.0 based on their sequence homology, allowing up to two mismatches in alignment [15]. Conserved miRNAs in common monocot and dicot species (*Triticum aestivum*, *Triticum turgidum*, *Brachypodium distachyon*, *Zea mays*, *Oryza sativa*, *Hordeum vulgare*, *Sorghum bicolor*, *Arabidopsis thaliana*, and *Glycine max*) deposited at miRBase v20 (June 2013) were used as references for annotation.

Normalisation of miRNA abundance in each library was carried out using a value referred to as RPM (reads per million). The RPM value was obtained by dividing the reads number of a miRNA with the total number of putative sRNA reads in each library and multiplying by a million. Matched sequences with no more than two mismatches and with an abundance of over two RPM in at least 50% of the 96 libraries were considered as candidate conserved miRNAs.

Identification of differentially expressed conserved miRNAs (Approach #1)

Differentially expressed conserved miRNAs were identified based on the RPM. To identify differentially expressed miRNAs, the following criteria were used: 1) number of miRNA reads was set as 0.01 by default when the sequencing read was 0; 2) normalised reads (RPM) was at least 10 in one of the libraries in comparison; and 3) the fold-change of normalised reads of libraries in comparison was greater than 1.5 [16,75]. For expression analysis, reads of unique mature miRNAs deposited in miRBase were used as they are an active and functional form of mature miRNAs [29]. Tissue-specific conserved miRNAs were identified by comparing flag leaf libraries with head libraries. Genotype-specific conserved miRNAs were identified by comparing water deficit sensitive varieties and water deficit tolerant varieties. Comparisons were made only between EGA Bellaroi and Tamaroi, or Tjilkuri and Yawa due to their breeding background. Water deficit stress-responsive miRNAs were identified by comparing control treatment libraries with water deficit stress treatment libraries. Heat maps of differentially expressed miRNAs were generated in R (version 3.1.2) (http://www.r-project.org/). Where the fold change of some conserved miRNA candidates were not analysed due to their low reads in the sequencing results (RPM were less than 10 in both libraries for differential expression comparison), their log2 fold change under stress was recorded as zero in the clustering analysis.

Small RNA-Seq data pre-processing for novel miRNA identification (Approach #2)

To identify novel miRNAs in durum wheat, a customised bioinformatics approach (Approach #2) was developed. Small RNA-Seq raw reads were 5' and 3' adapter trimmed and the output partitioned into two sets of reads: 1) those that had been trimmed and were 19–26 bp long, and 2) those that did not contain any adapter sequence. The first set represents non-redundant (NR) 3' adapter trimmed reads, which were used to identify putative pre-miRNA hairpin. In order to remove reads which are derived from the breakdown products of longer mRNA's rather than true sRNA molecules, the second set of reads and the NR sRNA reads were *de novo* assembled to generate a reference against which sRNA reads would be filtered. This was done using Velvet (v 1.2.09, https://www.ebi.ac.uk/~zerbino/velvet/) with a kmer length of 17 and read tracking enabled. The NR set of 3' adapter trimmed reads (19–26 bp) were filtered to remove those which were either: a) low abundance (< = 5 reads in all samples); b) mapped to known wheat rRNAs; c) mapped to the wheat chloroplast or mitochondrial genomes; d) mapped to the 50bp+ long *de novo* assembled contigs; e) mapped to UniVec (build 7.1, http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html) data set; or f) mapped to the Triticeae Repeat Sequence Database (TREP) database of grass repeat sequences [76]. The mappings in steps b-f above was performed using Bowtie2 (v 2.2.3; http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) with parameters which allowed up to two mismatches and no indels. The NR reads which passed all the above filters were used to identify candidate pre-miRNA hairpins.

Identification of miRNA precursors and novel miRNA candidates in durum wheat (Approach #2)

Since only a partial genome sequence for *Triticum turgidum* ssp. *durum* is available, the International Wheat Genome Sequencing Consortium's (IWGSC) Chromosomal Survey Sequences (CSS) [44] was used to identify putative miRNAs. The NR sRNA sequences which passed the filters were mapped to the IWGSC CSS using BioKanga v3.4.3 (http://sourceforge.net/projects/biokanga/) in order to identify all possible contigs from which the sRNA sequence could have been derived. For each NR 3' adapter trimmed read, all perfect alignment locations in the IWGSC CSS were identified. Using a subset of reads and CSS contigs involved in those perfect alignments, we also identified all imperfect alignments (two-five mismatches). The candidate pre-miRNA hairpins were defined using all pairwise combinations of perfect to imperfect alignments of a given read within a CSS contig. Additional constraints were applied such that the perfect and imperfect alignments were in opposite orientations and separated by 54–1000 bp. A NR set of these regions ±20 bp, were processed by RNAFold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) and then miRcheck (http://web.wi.mit.edu/bartel/pub/software.html) to ascertain if they could form hairpin structures with characteristics associated with the miRNA biogenesis pathway in plants, indicating the formation of a miRNA/miRNA* duplex from stem-loop hairpins based on their read coverage profile [55]. Three primary criteria were applied as follows: 1) A peak of reads in the first or last 50 bp of the hairpin sequence all aligned to the same strand/stem (the miRNA site); 2) a second, smaller peak of complementary reads aligned on the opposite end to the miRNA strand/stem (the miRNA* site); 3) a small proportion of reads mapping between the above two defined regions (the loop). All candidate miRNA hairpin sequences were classified into one of eight categories (A-H, where A has a read coverage profile matching the expectations for a true miRNA) using three Boolean metrics based on their read coverage profile: 1) if $\geq$95% of the reads mapped to one strand of the hairpin; 2) if $\geq$95% of the reads mapped to one of the terminal 50 bp of the hairpin; and; 3) if $\leq$5% of the reads mapped to the loop region of the hairpin (Table 2). Putative miRNA hairpins were further characterised by identifying if their sequence contained any perfect matches to the 35,828 mature miRNAs from miRBase v21 (accessed July 2014).

Identification of stress responsive novel miRNA hairpins (Approach #2)

To identify novel water deficit stress-responsive miRNA hairpins, the Limma Bioconductor (v3.18.13) package [45] was used to perform a statistical analysis using linear models based on the RPM data. Different durum varieties were recoded with binary values which indicated water deficit stress tolerance or water deficit stress sensitivity. Of the many possible contrasts that could be made, the tolerance × treatment interaction term was of primary interest in the linear model. This effectively identified hairpins which showed differential expression to water deficit stress and that this response was different between water deficit stress sensitive and water deficit stress tolerant cultivars. Pre-miRNA hairpins from Category A (Table 2) which had a significant tolerance × treatment interaction were then inspected to ascertain if their read-coverage profiles followed what we expected from a true mature miRNA and miRNA*.

Quantitative real-time PCR (qPCR) of miRNA candidates

In order to evaluate the expression of miRNA candidates, poly-A tailing combined with qPCR was performed for a select group of seven conserved and three novel stress responsive miRNAs with the 96 durum total RNA samples which were used for sRNA library construction. For each sample, 1 μg of total RNA was poly-A tailed and reverse-transcribed with the NCode

VILO miRNA cDNA synthesis kit (Invitrogen, USA) following the manufacturer's instructions. The final cDNA product was diluted to 100 μL. qPCR was performed using the ViiA™ 7 Real-Time PCR system (Applied Biosystems, USA). In each 10 μL qPCR reaction (six biological replicates for each sample), 1 μL diluted cDNA template and primers (3 pmol of each forward and reverse) were mixed with SYBR® Green reagent (iQ ™ supermix, BioRad, USA). The forward miRNA primers were designed based on the full mature miRNA sequences (S9 Table). The reverse primer was the universal reverse primer provided in the NCode VILO miRNA cDNA synthesis kit. The qPCR running conditions were: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 56/58/60°C for 15 s, and 70°C for 10 s, followed by 72°C for 10 min. Melting curve analysis was used to detect the specificity of the amplified product. The relative expression ratio was calculated using the comparative CT ($^{\Delta\Delta}C_T$) method with GAPDH [GenBank: AF251217] as the reference gene.

Target prediction, functional GO analysis and target qPCR

The putative mRNA targets of stress responsive novel miRNAs were identified using psRNA Target Server (http://plantgrn.noble.org/psRNATarget/) with the following parameters: prediction score cut-off value = 3.0, length for complementarity scoring = 20, and target accessibility = 25. Mature novel miRNA sequences were used as queries and the wheat DFCI gene index (TAGI) version 12 was used as the reference genome dataset [19]. All the predicted targets were evaluated using the functional enrichment analysis tool at Blast2GO (http://www.blast2go.com) [77,78]. BLASTX was employed to perform a homology search against the NR protein databases in NCBI to obtain the most significant BLAST hits for each target using the Blast function with Blast2GO. Default parameters were used in the mapping and annotation steps to obtain GO terms for each target transcript in Blast2GO. The annotation results were further improved by analysing conserved domains/families using the InterProScan function. GO terms for three categories (cellular component, molecular function and biological processes) were determined for each annotated target. All the annotated targets were classified on the basis of their GO term enrichments in each category. Seven selected functional targets were quantified using qPCR with the same cDNA libraries employed in the miRNA qPCR. Target qPCR was performed using the comparative CT ($^{\Delta\Delta}C_T$) method with GAPDH as the reference gene [GenBank: AF251217]. Target primers were designed to include the predicted miRNA/mRNA binding region in the amplified product ensuring the quantification of uncleaved targets, in order to examine the correlation of miRNA and regulated targets. Target transcript sequences, primer locations and primer sequences are listed in S10 Table.

Supporting Information

S1 Fig. The length distribution of small RNA reads obtained by high-throughput sequencing in durum wheat. Only one representative library (from a total of 96 libraries) is shown. All sequencing reads were submitted to the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/), and are accessible under the accession number GSE69339.
(EPS)

S2 Fig. Predicted secondary structures of five novel durum miRNA hairpins that are responsive to water deficit stress. Mature miRNAs are highlighted in blue while miRNA* are highlighted yellow. The secondary structures of the novel durum wheat miRNA hairpins (A) Ttu-pre-miR007, (B) Ttu-pre-miR008, (C) Ttu-pre-miR038, (D) Ttu-pre-miR109, and (E) Ttu-pre-miR119 are shown.
(TIF)

S3 Fig. Species distribution of all BLAST hit alignments from the GO analysis. Identified target gene transcripts are searched in the species-specific entries registered in the GO database. Species distribution is based on the number of BLAST hits aligned in each species.
(TIF)

S1 Table. Sequencing reads and the output data obtained from the CLC Genomics workbench pipeline. Data is shown for 96 libraries presented in 16 different biological library pools (four genotypes × two tissue types × two treatments). CG = Control group; WG = Water deficit stress group; FL = Flag leaf libraries; H = Head libraries.
(XLS)

S2 Table. List of known microRNAs in durum wheat and their normalised reads in each library. CG = Control group; WG = Water deficit stress group; FL = Flag leaf libraries; H = Head libraries.
(XLS)

S3 Table. List of novel durum microRNA hairpins and their normalised reads in each library. CG = Control group; WG = Water deficit stress group; FL = Flag leaf libraries; H = Head libraries. The Hairpin Alignment Identifier is derived from the genome location information of the hairpin sequence in the IWGSC CSS (International Wheat Genome Sequencing Consortium's Chromosomal Survey Sequences), as well as the alignment position and the length of the reads used to identify putative microRNA hairpins. The Hairpin Alignment Identifiers take the following form as an example: 1AL_3896362:3010–3120[21,21]. 1AL_3896362 = the sequence identifier in the IWGSC CSS, this is from chromosomal arm 1AL; 3010 = position of the first base of the hairpin within the IWGSC CSS; 3120 = position of the last base of the hairpin within the IWGSC CSS; [21,21] = the putative mature miRNA starts at position 21 in the hairpin and is 21 bp in length.
(XLS)

S4 Table. Conserved water deficit stress responsive miRNAs in durum wheat. Fold changes have been determined by comparing the RPM between the control treatment libraries and the water deficit stress treatment libraries in the flag leaf and the developing head of four durum wheat genotypes. Fold changes are shown when greater than 1.5 fold. Green values indicate that miRNA reads were more abundant in the water deficit treatment libraries. Red values indicate that the miRNA reads were more abundant in the control treatment libraries.
CG = Control group; WG = Water deficit stress group; FL = Flag leaf libraries; H = Head libraries; Be = EGA Bellaroi; Ta = Tamaroi; Tj = Tjilkuri; Ya = Yawa; Ath = *Arabidopsis thaliana*; Bdi = *Brachypodium distachyon*; Gma = *Glycine max*; Hvu = *Hordeum vulgare*; Osa = *Oryza sativa*; Sbi = *Sorghum bicolor*; Tae = *Triticum aestivum*; Zma = *Zea mays*.
(XLS)

S5 Table. Fold-change of selected water deficit stress responsive miRNA candidates identified by Illumina sequencing and qPCR. Fold changes have been determined by comparing the RPM in Illumina sequencing or comparing relative expression ratio in qPCR between the control treatment and the water deficit stress treatment in different tissues of four durum wheat genotypes. Bold fold change value indicates that the miRNA was more abundant in the water deficit stress treatment libraries whereas unbolded fold change indicates that the miRNA was more abundant in the control treatment libraries. FL = Flag leaf libraries; H = Head libraries; CG = Control group; WG = Water deficit stress group; Be = EGA Bellaroi; Ta = Tamaroi; Tj = Tjilkuri; Ya = Yawa; Ath = *Arabidopsis thaliana*; Bdi = *Brachypodium distachyon*; Gma =

Glycine max; Osa = *Oryza sativa*; Zma = *Zea mays*.
(XLS)

S6 Table. Predicted targets of four novel durum stress responsive miRNAs and their GO analysis results. Definitions: Column E (Expectation)–The expectation scoring of the complementarity between miRNAs and their targets. The maximum expectation threshold score was set at 3.0. Column F (Target Accessibility (UPE))–The maximum energy required to open (unpair) the secondary structure around the target site on the target mRNA. Column O (Multiplicity)–Multiplicity of the target site representing the number of target sites within a specific target transcript.
(XLS)

S7 Table. Combined Gene Ontology classification in GO levels of 130 predicted targets of four novel miRNAs. Definitions: Column A (Level)–The GO level represents the position of a GO term in the GO hierarchy. The level of a GO term is the number of GO terms between that term and the Root Term of the Ontology. Column E (Node score)–The node score is the sum of sequences directly or indirectly associated to a given GO term weighted by the distance of this term to the term of its direct annotation, i.e. the GO term the sequence is originally annotated to. This confluence score takes into account the number of sequences converging at one GO term and at the same time penalises by the distance to the term where each sequence was actually annotated. Column F (%Seq)–The percentage of sequences annotated with a particular GO term among all the sequences annotated within the same GO level. Column G (#Seq)–The number of target sequences annotated with that particular GO term.
(XLS)

S8 Table. Fold-change of seven selected functional targets of Ttu-miR008 quantified by qPCR. Green values indicate that the targets were up-regulated under water deficit stress, while red values indicate that the targets were down-regulated under water deficit stress. Bold fold change values indicate negative correlation with Ttu-miR008. FL = Flag leaf libraries; H = Head libraries.
(XLS)

S9 Table. Forward primers used in qPCR validation of seven conserved and three novel miRNAs in durum. Each forward primer was designed based on the full sequence of the mature miRNA.
(XLS)

S10 Table. Target transcript sequences, primer locations and primer sequences used in qPCR validation of seven selected target genes.
(XLS)

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Author Contributions

Conceived and designed the experiments: HL IRS DEM AJA JAA. Performed the experiments: HL. Analyzed the data: HL NSW-H UB AJA JAA. Contributed reagents/materials/analysis tools: HL IRS NSW-H UB AJA JAA. Wrote the paper: HL IRS NSW-H UB DEM AJA JAA.

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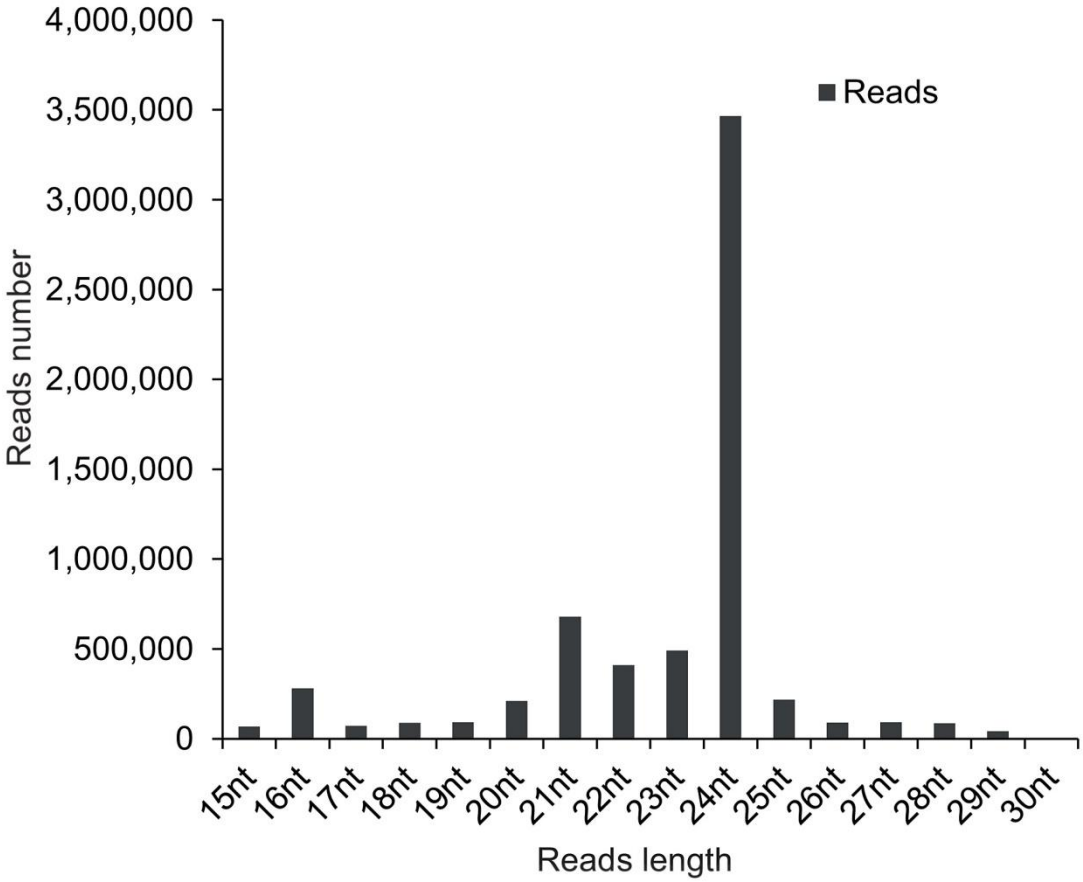
Chapter 4 Addendum

Supplementary materials available online via DOI

S1 Fig. The length distribution of small RNA reads obtained by high-throughput sequencing in durum wheat.

Only one representative library (from a total of 96 libraries) is shown. All sequencing reads were submitted to the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/), and are accessible under the accession number GSE69339.

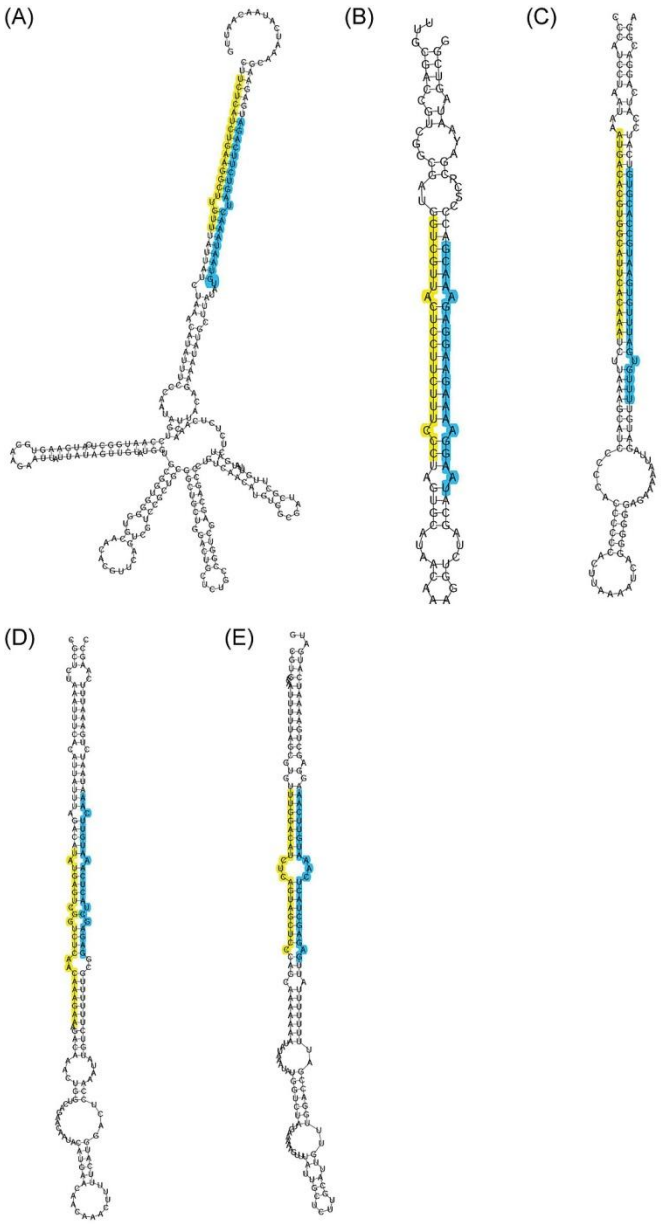
http://dx.doi.org/10.1371/journal.pone.0142799.s001



S2 Fig. Predicted secondary structures of five novel durum miRNA hairpins that are responsive to water deficit stress.

Mature miRNAs are highlighted in blue while miRNA* are highlighted yellow. The secondary structures of the novel durum wheat miRNA hairpins (A) Ttu-pre-miR007, (B) Ttu-pre-miR008, (C) Ttu-pre-miR038, (D) Ttu-pre-miR109, and (E) Ttu-pre-miR119 are shown.

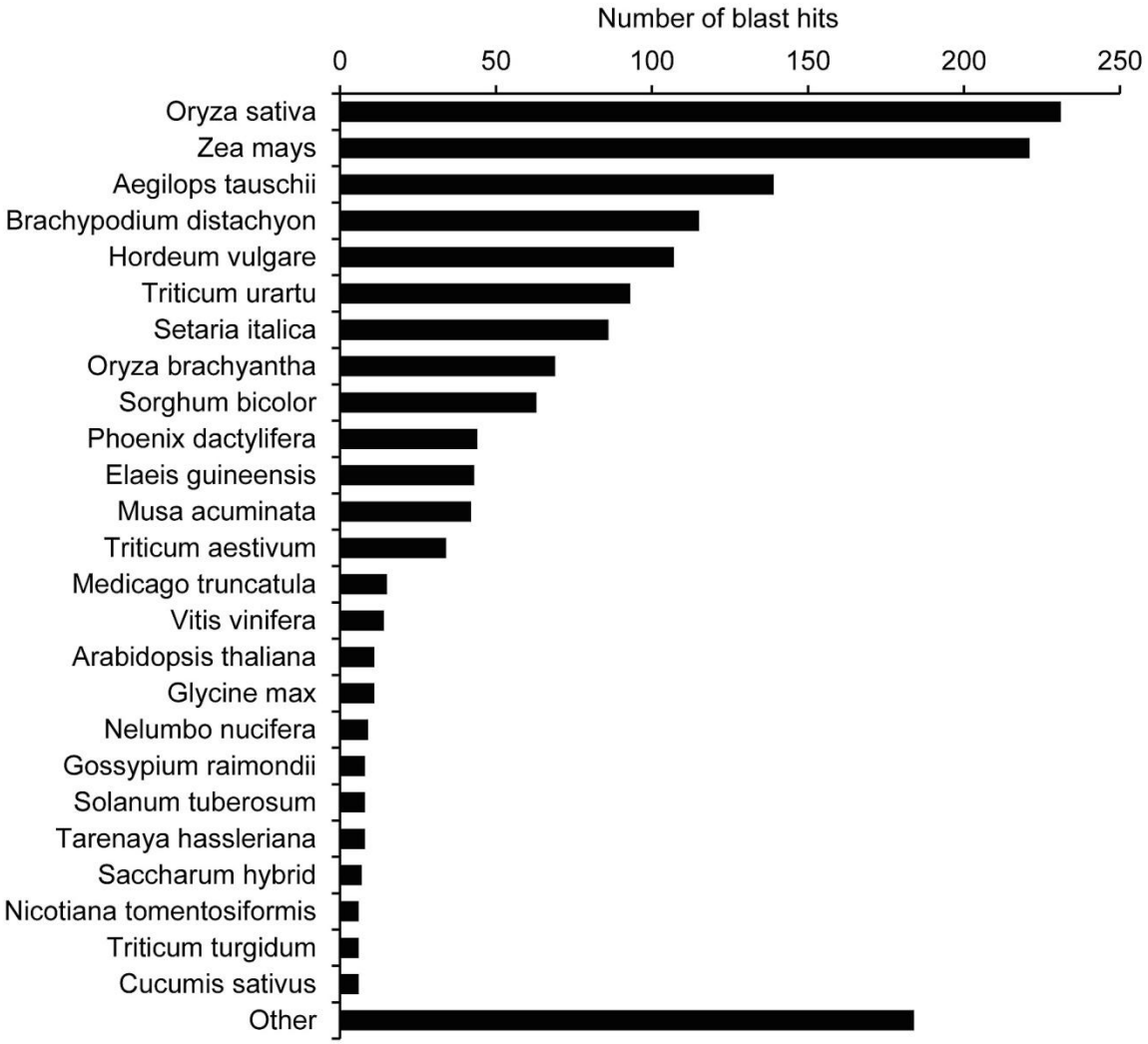
http://dx.doi.org/10.1371/journal.pone.0142799.s002



S3 Fig. Species distribution of all BLAST hit alignments from the GO analysis.

Identified target gene transcripts are searched in the species-specific entries registered in the GO database. Species distribution is based on the number of BLAST hits aligned in each species.

http://dx.doi.org/10.1371/journal.pone.0142799.s003



S1 Table. Sequencing reads and the output data obtained from the CLC Genomics workbench pipeline.

Data is shown for 96 libraries presented in 16 different biological library pools (four genotypes × two tissue types × two treatments). CG = Control group; WG = Water deficit stress group; FL = Flag leaf libraries; H = Head libraries.

http://dx.doi.org/10.1371/journal.pone.0142799.s004

Genotype	Tissue	Treatment	Total number of reads	Reads after trimming	Average length after trimming	Number of unique sRNA reads	Total reads of annotated sRNAs	Number of annotated sRNAs
Tamaroi	FL	CG	37,525,876	35,139,774	22.5	16,074,839	1,711,773	133,679
		WG	47,767,962	45,947,175	23	20,459,463	2,263,214	165,383
	H	CG	37,547,192	35,887,079	23.1	19,448,502	941,518	96,181
		WG	26,792,282	25,057,445	22.9	13,449,378	561,116	72,942
Yawa	FL	CG	40,144,676	38,844,900	23.3	19,246,128	1,765,155	159,982
		WG	39,897,309	38,748,830	23.2	18,364,404	1,624,811	145,733
	H	CG	34,235,504	32,895,880	23.4	19,337,657	822,541	100,574
		WG	25,062,063	24,461,794	23.5	12,943,859	391,049	57,862
EGA Bellaroi	FL	CG	47,297,911	46,174,489	22.9	21,968,247	2,254,376	167,914
		WG	28,097,531	27,501,751	23	14,606,371	1,423,301	131,499
	H	CG	55,957,642	53,976,273	23	23,872,847	948,082	105,245
		WG	15,048,062	17,194,123	22.9	9,933,861	378,947	55,635
Tjilkuri	FL	CG	40,851,763	38,784,249	23.1	17,865,003	1,704,205	142,986
		WG	57,354,547	55,419,283	22.9	26,950,939	2,745,487	202,523
	H	CG	24,668,244	23,854,494	23.4	14,425,720	533,510	75,115
		WG	65,122,199	62,212,585	23.3	32,905,027	1,511,898	147,223

S2 Table. List of known microRNAs in durum wheat and their normalised reads in each library.

CG = Control group; WG = Water deficit stress group; FL = Flag leaf libraries; H = Head libraries.

*Please click on the following DOI link or scan the QR code to download this supplementary material. The size of this table is not suitable for thesis binding.

http://dx.doi.org/10.1371/journal.pone.0142799.s005

S3 Table. List of novel durum microRNA hairpins and their normalised reads in each library.

CG = Control group; WG = Water deficit stress group; FL = Flag leaf libraries; H = Head libraries. The Hairpin Alignment Identifier is derived from the genome location information of the hairpin sequence in the IWGSC CSS (International Wheat Genome Sequencing Consortium's Chromosomal Survey Sequences), as well as the alignment position and the length of the reads used to identify putative microRNA hairpins. The Hairpin Alignment Identifiers take the following form as an example: 1AL_3896362:3010–3120[21,21]. 1AL_3896362 = the sequence identifier in the IWGSC CSS, this is from chromosomal arm 1AL; 3010 = position of the first base of the hairpin within the IWGSC CSS; 3120 = position of the last base of the hairpin within the IWGSC CSS; [21,21] = the putative mature miRNA starts at position 21 in the hairpin and is 21 bp in length.

*Please click on the following DOI link or scan the QR code to download this supplementary material. The size of this table is not suitable for thesis binding.

http://dx.doi.org/10.1371/journal.pone.0142799.s006

S4 Table. Conserved water deficit stress responsive miRNAs in durum wheat.

Fold changes have been determined by comparing the RPM between the control treatment libraries and the water deficit stress treatment libraries in the flag leaf and the developing head of four durum wheat genotypes. Fold changes are shown when greater than 1.5 fold. Green values indicate that miRNA reads were more abundant in the water deficit treatment libraries. Red values indicate that the miRNA reads were more abundant in the control treatment libraries. CG = Control group; WG = Water deficit stress group; FL = Flag leaf libraries; H = Head libraries; Be = EGA Bellaroi; Ta = Tamaroi; Tj = Tjilkuri; Ya = Yawa; Ath = *Arabidopsis thaliana*; Bdi = *Brachypodium distachyon*; Gma = *Glycine max*; Hvu = *Hordeum vulgare*; Osa = *Oryza sativa*; Sbi = *Sorghum bicolor*; Tae = *Triticum aestivum*; Zma = *Zea mays*.

*Please click on the following DOI link or scan the QR code to download this supplementary material. The size of this table is not suitable for thesis binding.

http://dx.doi.org/10.1371/journal.pone.0142799.s007

S5 Table. Fold-change of selected water deficit stress responsive miRNA candidates identified by Illumina sequencing and qPCR.

Fold changes have been determined by comparing the RPM in Illumina sequencing or comparing relative expression ratio in qPCR between the control treatment and the water deficit stress treatment in different tissues of four durum wheat genotypes. Bold fold change value indicates that the miRNA was more abundant in the water deficit stress treatment libraries whereas unbolded fold change indicates that the miRNA was more abundant in the control treatment libraries. FL = Flag leaf libraries; H = Head libraries; CG = Control group; WG = Water deficit stress group; Be = EGA Bellaroi; Ta = Tamaroi; Tj = Tjilkuri; Ya = Yawa; Ath = *Arabidopsis thaliana*; Bdi = *Brachypodium distachyon*; Gma = *Glycine max*; Osa = *Oryza sativa*; Zma = *Zea mays*.

http://dx.doi.org/10.1371/journal.pone.0142799.s008

Name	Resource species in miRBase	Flag leaf							
		WG/CG							
		Sequencing				qPCR			
		Be	Tj	Ta	Ya	Be	Tj	Ta	Ya
miR167d	Ath	1.319	2.633	**1.535**	**1.218**	1.043	2.802	**1.552**	**3.373**
miR408d	Gma	**2.097**	**1.632**	**1.444**	**2.293**	**1.349**	**1.261**	**1.582**	**2.567**
miR5054	Bdi	1.349	**1.013**	1.564	2.292	**1.882**	**1.989**	1.505	1.505
miR5071	Osa	**1.171**	**1.073**	1.109	1.302				
miR5200	Bdi	**2.768**	13.822	**1.259**	1.511	**2.508**	21.422	**1.271**	1.472
miR528	Bdi	**8.568**	**1.687**	**5.535**	1.715	**5.185**	**1.080**	**2.804**	1.480
miR528a	Zma	**2.847**	**1.528**	**1.216**	1.151	**2.271**	**1.215**	**1.893**	**1.191**
		Head							
		WG/CG							
		Sequencing				qPCR			
		Be	Tj	Ta	Ya	Be	Tj	Ta	Ya
miR167d	Ath	**1.177**	2.736	1.124	1.511	**1.178**	1.896	1.155	**1.033**
miR408d	Gma	**8.810**	1.113	5.164	1.526	**1.610**	2.255	4.914	1.359
miR5054	Bdi	**1.166**		1.059	1.574	**1.722**	2.603	2.140	1.037
miR5071	Osa	**1.514**	**1.141**	**1.057**	1.965	**1.304**	**1.409**	1.162	1.145
miR5200	Bdi	**2.853**	7.766	1.544	3.128	**2.121**	8.191	1.496	1.070
miR528	Bdi			2.237		**1.565**	2.315	1.292	**1.253**
miR528a	Zma	**3.587**	1.216	5.106	2.748	**2.163**	2.578	4.937	1.582

S6 Table. Predicted targets of four novel durum stress responsive miRNAs and their GO analysis results.

Definitions: Column E (Expectation)–The expectation scoring of the complementarity between miRNAs and their targets. The maximum expectation threshold score was set at 3.0. Column F (Target Accessibility (UPE))–The maximum energy required to open (unpair) the secondary structure around the target site on the target mRNA. Column O (Multiplicity)–Multiplicity of the target site representing the number of target sites within a specific target transcript.

*Please click on the following DOI link or scan the QR code to download this supplementary material. The size of this table is not suitable for thesis binding.

http://dx.doi.org/10.1371/journal.pone.0142799.s009

S7 Table. Combined Gene Ontology classification in GO levels of 130 predicted targets of four novel miRNAs.

Definitions: Column A (Level)–The GO level represents the position of a GO term in the GO hierarchy. The level of a GO term is the number of GO terms between that term and the Root Term of the Ontology. Column E (Node score)–The node score is the sum of sequences directly or indirectly associated to a given GO term weighted by the distance of this term to the term of its direct annotation, i.e. the GO term the sequence is originally annotated to. This confluence score takes into account the number of sequences converging at one GO term and at the same time penalises by the distance to the term where each sequence was actually annotated. Column F (%Seq)–The percentage of sequences annotated with a particular GO term among all the sequences annotated within the same GO level. Column G (#Seq)–The number of target sequences annotated with that particular GO term.

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http://dx.doi.org/10.1371/journal.pone.0142799.s010

S8 Table. Fold-change of seven selected functional targets of Ttu-miR008 quantified by qPCR.

Green values indicate that the targets were up-regulated under water deficit stress, while red values indicate that the targets were down-regulated under water deficit stress. Bold fold change values indicate negative correlation with Ttu-miR008. FL = Flag leaf libraries; H = Head libraries.

http://dx.doi.org/10.1371/journal.pone.0142799.s011

Target	Description	FL EGA Bellaroi	FL Tjilkuri	FL Tamaroi	FL Yawa	H EGA Bellaroi	H Tjilkuri	H Tamaroi	H Yawa
CV779294	non-specific lipid-transfer protein a-like	**2.367**	**2.411**	**1.342**	**1.402**	1.265	1.266	1.065	1.966
TC438017	non-specific lipid-transfer protein 1	**2.718**	**1.107**	**4.258**	**2.789**	6.160	**1.334**	1.040	**1.413**
TC372193	phytoene synthase 2	1.763	**1.957**	1.994	**1.117**	**1.885**	**1.526**	**1.240**	**1.419**
TC447684	glossy1 protein	1.902	1.297	1.475	2.328	**1.172**	**1.520**	**1.224**	**1.129**
CD904770	cycteine-rich receptor-like protein kinase 25	1.681	**2.560**	1.970	**1.755**	**1.149**	1.712	**1.301**	5.842
TC411916	phytoene synthase 2	1.016	**1.080**	1.159	**1.427**	2.868	**1.385**	4.646	**1.102**
TC409543	l-ascorbate oxidase homolog	**1.295**	1.369	**4.554**	2.072	**1.279**	1.711	**1.483**	1.044

S9 Table. Forward primers used in qPCR validation of seven conserved and three novel miRNAs in durum.

Each forward primer was designed based on the full sequence of the mature miRNA.

http://dx.doi.org/10.1371/journal.pone.0142799.s012

	Mature miRNA sequence	Name	Forward primer sequence ('5 to '3)	Tm (°C)
1	TGAAGCTGCCAGCATGATCTGG	Ath-miR167d	GAAGCTGCCAGCATGATCTGG	58
2	TGCACTGCCTCTTCCCTGGC	Gma-miR408d	TATAGCCTGCACTGCCTCTTC	58
3	TCCCCACGGTCGGCGCCA	Bdi-miR5054	TATTATCCCCACGGTCGGCG	60
4	TCAAGCATCATATCGTGGACA	Osa-miR5071	GGTCAAGCATCATATCGTGGAC	60
5	TGTAGATACTCTCTAAGGCTT	Bdi-miR5200	GCGTGTAGATACTCTCTAAGGCTT	60
6	CCTGTGCCTGCCTCTTCCATT	Bdi-miR528	CTGTGCCTGCCTCTTCCATT	60
7	TGGAAGGGGCATGCAGAGGAG	Zma-miR528a	TATACTGGAAGGGGCATGCAGA	58
8	TGTAATAAACTAGTCTTCAGA	Ttu-miR007	GCGGTGTAATAAACTAGTCTTCAGA	56
9	TTTGTGATTTGTGAATGCCACGTG	Ttu-miR038	TTTGTGATTTGTGAATGCCACGTG	56
10	GAGAGCTACTCAAATGTTCAA	Ttu-miR109	GCCGAGAGCTACTCAAATGTTCAA	56

S10 Table. Target transcript sequences, primer locations and primer sequences used in qPCR validation of seven selected target genes.

*Please click on the following DOI link or scan the QR code to download this supplementary material. The size of this table is not suitable for thesis binding.

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Chapter 5

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Haipei Liu		
Contribution to the Paper	Designed the experiments, conducted the research, analysed the data and drafted the manuscript.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	22/08/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Amanda Able		
Contribution to the Paper	Designed the experiments, analysed the data and drafted the manuscript.		
Signature		Date	22/08/16

Name of Co-Author	Jason Able		
Contribution to the Paper	Designed the experiments, analysed the data, drafted the manuscript and acted as the corresponding author.		
Signature		Date	22/08/16

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ORIGINAL ARTICLE

Water-deficit stress-responsive microRNAs and their targets in four durum wheat genotypes

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Response to Q1:Yes all author's name are correct.

Please note that this is the Author's Proof version. All text boxes highlighted in red indicate Author's corrections.

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Abstract MicroRNAs (miRNAs) guide regulation at the post-transcriptional level by inducing messenger RNA (mRNA) degradation or translational inhibition of their target protein-coding genes. Durum wheat miRNAs may contribute to the genotypic water-deficit stress response in different durum varieties. Further investigation of the interactive miRNA-target regulatory modules and experimental validation of their response to water stress will contribute to our understanding of the small RNA-mediated molecular networks underlying stress adaptation in durum wheat. In this study, a comprehensive genome-wide in silico analysis using the updated *Triticum* transcriptome assembly identified 2055 putative targets for 113 conserved durum miRNAs and 131 targets for four novel durum miRNAs that putatively contribute to genotypic stress tolerance. Predicted mRNA targets encode various transcription factors, binding proteins and functional enzymes, which play vital roles in multiple biological pathways such as hormone signalling and metabolic processes. Quantitative PCR profiling further characterised 43 targets and 5 miRNAs with stress-responsive and/or genotype-dependent differential expression in two stress-tolerant and two sensitive durum genotypes subjected to pre-anthesis water-deficit stress. Furthermore, a 5′ RLM-RACE approach validated nine mRNA targets cleaved by water-deficit stress-responsive miRNAs, which, to our knowledge, has not been previously reported in durum wheat. The present study provided experimental evidence of durum miRNAs and target genes in response to water-deficit stress in contrasting durum varieties, providing new insights into the regulatory roles of the miRNA-guided RNAi mechanism underlying stress adaptation in durum wheat.

two stress-sensitive

Keywords Durum wheat · microRNAs · mRNA targets · Water-deficit stress response

water-deficit

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Response to Q2: Yes this information is all correct.

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Introduction

Stress-tolerant crop varieties exhibit environmentally adaptive traits (both physiological and morphological) that enable the plant to endure stressful conditions and achieve reproductive success under unfavourable environments (Dolferus 2014; Liu et al. 2015b). Research efforts to dissect the sophisticated molecular mechanisms that underlie adaptive traits have identified many novel regulatory players including microRNAs (miRNAs). The biogenesis, organisation and functions of plant miRNAs have been summarised in several recent reviews (Borges and Martienssen 2015; Budak and Akpinar 2015; Wang and Chekanova 2016; Zhang 2015; Zhang and Wang 2015). Briefly, mature plant miRNAs are single-stranded endogenous RNA molecules of 20–25 nucleotides (nt) in length, which are precisely processed from their longer stem-loop precursors (pre-miRNA hairpins) transcribed from the *MIR* genes encoded mainly in the intergenic regions of the genome (Vazquez et al. 2010). MicroRNA is incorporated into the RNA-induced silencing complex (RISC) in the RNA silencing mechanism, suppressing their target protein-coding genes at the post-transcriptional

level (Borges and Martienssen 2015). In the RISC, miRNA acts as the guiding molecule and binds to its imperfect complementary sequence within the cognate messenger RNA (mRNA) targets, inducing either cleavage degradation or translational inhibition (Jones-Rhoades et al. 2006; Sunkar et al. 2007).

Emerging evidence in many model and complex plant species has revealed the key regulatory roles of miRNAs in plant development, reproduction and stress responses (Ferdous et al. 2016; Gao et al. 2015; Jiao et al. 2010; Liu et al. 2015b; Rubio-Somoza and Weigel 2013; Sun et al. 2012; Wang et al. 2013; Wang et al. 2015; Xie et al. 2015a). Various miRNA families could respond to and integrate both environmental and developmental cues, reprogramming numerous downstream gene transcription events implicated in the biological processes contributing to plant fitness and survival (Budak et al. 2015b; Sunkar et al. 2012; Wang and Chekanova 2016; Zhang 2015). Such processes under the tight control of miRNAs include leaf elongation, lateral root formation, tiller development, floral transition, floral organ separation, reproductive branching and fruit/grain development (Bertolini et al. 2013; Cao et al. 2016; Gao et al. 2015; Rubio-Somoza and Weigel 2013; Wang et al. 2015; Xia et al. 2012, 2015a; Xu et al. 2014). By primarily targeting transcription factors in multiple signal transduction pathways including those that involve abscisic acid, auxin, gibberellin and jasmonic acid, miRNA can control stress adaptation and plant development (Curaba et al. 2014). Thus, miRNAs are crucial to plant defence against environmental abiotic stresses and plant development.

In the effort to explore the potential use of miRNAs in the genetic improvement of stress tolerance, a number of studies have been conducted in agronomically important cereal species to identify stress-associated miRNAs and their functional targets (Budak and Akpinar 2011; Budak et al. 2015b, c; Gupta et al. 2014; Hackenberg et al. 2015; Kantar et al. 2010; Liu et al. 2015b; Ma et al. 2015). For example, recent assessments in bread wheat and its progenitors have revealed many miRNAs associated with abiotic stress in various genotypes (Agharbaoui et al. 2015; Alptekin and Budak 2016; Budak and Bala Ani 2016; Eren et al. 2015; Gupta et al. 2014; Kumar et al. 2014; Kurtoglu et al. 2013; Ma et al. 2015; Pandey et al. 2014) that could be exploited via RNA interference (RNAi) technologies in developing elite varieties (Budak et al. 2013, 2015a). However, attention paid to durum wheat (*Triticum turgidum* L. ssp. *durum*, AABB, $2n = 4\times = 28$) has been limited, despite its agronomic importance as the most cultivated tetraploid wheat, especially under Mediterranean environments. In Mediterranean environments, like South Australia, water-deficit stress that occurs during early plant reproductive stages is the major cause of grain number reduction and yield loss in durum (Liu et al. 2015a). Using Illumina sequencing, we have previously identified conserved and novel durum miRNAs that were responsive to pre-anthesis water-deficit stress in a genotypic-dependent manner and predicted putative target genes for four of the novel miRNAs (Liu et al. 2015b). Previous studies investigating the miRNA repertoire of wild emmer and modern durum wheat have also identified *T. turgidum* miRNAs associated with drought response, with some of their putative targets predicted (Akpinar et al. 2015; Kantar et al. 2011). Nevertheless, to date, very limited experimental evidence has been provided for the target genes of durum miRNAs and their interactions in response to water-deficit stress. Further investigation and experimental validation of durum stress-responsive miRNAs and their functional target genes will provide new insights into the miRNA-mediated regulatory pathways underlying water stress tolerance in different durum varieties.

In this study, genome-wide in silico analysis of the target transcripts of durum miRNAs was performed to predict their possible functional roles in water-deficit stress response and plant development. The target repertoire of stress-responsive durum miRNAs includes a broad range of proteins related to stress perception, phytohormone signal transduction and metabolic processes. Subsequent quatitative polymerase chain reaction (qPCR) profiling of 43 targets and five miRNAs in stress-tolerant (Tamaroi and Yawa) and sensitive (EGA Bellaroi and Tjilkuri) durum varieties revealed differential expression patterns associated with stress treatments, tissue types and genotypes. 5′ RLM-RACE further validated the post-transcriptional gene silencing of nine target genes through miRNA-guided cleavage. This study therefore systematically predicted durum miRNA-targeted functional genes and provides experimental evidence of durum miRNA/target interactions upon pre-anthesis water-deficit stress.

and -sensitive

Methods

Plant materials and total RNA extraction

water-deficit stress-sensitive

For the four durum wheat varieties used in this study, Tamaroi and Yawa are water-deficit stress-tolerant genotypes, while EGA Bellaroi and Tjilkuri are water-deficit stress sensitive (Liu et al. 2015a). Durum seeds were provided by Durum Breeding Australia's (DBA) southern node breeding program (The University of Adelaide). Plants were grown under glasshouse conditions at 22 °C/12 °C day/night temperature with a 12-h photoperiod as previously described (Liu et al. 2015b). Briefly, durum wheat seedlings were well-watered to field capacity (12 % soil water content (SWC)) from germination to booting stage. At booting, plants in the water-deficit stress group (WG) had the soil water content (SWC) maintained at 6 % for 15 days from booting, while the control group (CG) continued to be well watered (Liu et al. 2015b). Flag leaf and the developing head were sampled at 15 days after booting. A total of 96 samples from four durum genotypes, two different tissues (flag leaf and head), two treatments (CG and WG) with

six biological replicates were collected and frozen immediately in liquid nitrogen and stored at −80 °C for further use. Total RNA samples were isolated with the TriPure reagent (Roche Diagnostics, Australia) and treated with RQ1 RNase-Free DNase I (Promega, Australia) following the manufacturer's instructions. The concentration and quality of total RNA samples were measured by spectrophotometric analysis at 260 and 280 nm using a NanoDrop Lite spectrophotometer (Thermo Scientific, USA). High quality RNA, as assessed by electrophoresis on a 2 % agarose gel, was used for qPCR analysis.

Target prediction and functional annotation

In this study, the approach combining in silico miRNA target prediction and experimental validation is schematically represented in Fig. 1. The Web-based psRNATarget server (http://plantgrn.noble.org/psRNATarget/) was employed for target prediction with default parameters as described (Akpinar et al. 2015; Liu et al. 2015b). A total of 69 conserved stress-responsive durum miRNAs, 4 novel stress-responsive durum miRNAs and 44 other conserved durum miRNAs identified previously in the same four durum wheat genotypes (Liu et al. 2015b) were used as queries in the target prediction (Fig. 1) using an updated version of the *Triticum* assembly including non-redundant sets of *Triticum aestivum* and *T. turgidum* transcriptomes (Krasileva et al. 2013) as the reference dataset. The Gene Ontology (GO) annotation of all target genes was performed using the Blast2GO software (version 3.2; http://www.blast2go.com) (Conesa and Gotz 2008). All candidate target sequences were imported into the Blast2GO program and the following four steps were performed: (1) sequence homology blast search against the NCBI non-redundant protein database using the BLASTx suite; (2) sequence mapping to retrieve the GO terms associated with the BLASTx results; (3) sequence annotation to select the most reliable gene functions associated with the mapped GO terms; and (4) improvement of GO annotation by analysing conserved domains/families using the built-in InterProScan function. GO terms at level 4 for cellular components and level 3 for molecular functions and biological processes were used to generate GO classification pie charts.

Stress-responsive miRNA and target validation with qRT-PCR

In order to validate the in silico-predicted mRNA targets and to investigate their gene expression in response to water-deficit stress, qPCR experiments were performed in four durum wheat varieties with contrasting stress tolerance. A total of 96 poly(A)-tailed complementary DNA (cDNA) samples, made from the 96 total RNA samples (from four durum genotypes × two different tissues × two treatments × six biological replicates), were synthesised using the NCode VILO miRNA cDNA synthesis kit (Invitrogen, USA) according to manufacturer's instructions as previously described (Liu et al. 2015b). Expression profiles of 43 target candidates of interest (Table 1) and five conserved stress-responsive durum miRNAs (Ath-miR160a, Sbi-miR160f, Bdi-miR167e, Ath-miR396b, Bdi-miR827-3p) were quantified using SYBR Green reagent (iQ TM supermix, BioRad, USA) on a ViiA™ 7 Real-Time PCR machine (Applied Biosystems, USA) in these 96 cDNA samples. For the amplification of mRNA targets, forward and reverse primers were designed to include the predicted miRNA/target binding region in qPCR products, ensuring the quantification of uncleaved target transcripts (Electronic supplementary material Table S1). For the amplification of miRNAs, forward miRNA-specific primers were designed based on the full mature miRNA sequences (Electronic Supplementary Material Table S2) and the universal adaptor-specific reverse primer was provided in the NCode VILO miRNA cDNA synthesis kit. Melting curves were performed and evaluated at the end of each qPCR reaction to ensure specificity. The comparative CT ($^{\Delta\Delta}$CT) method was used to calculate the relative expression of miRNAs and mRNA targets with GAPDH as the reference gene (Liu et al. 2015b), which was previously confirmed to be stably expressed in the durum wheat tissues used (data not shown).

Validation of cleaved target fragments with 5′ RLM-RACE

To validate computationally predicted targets of interest, a modified version of 5′ RLM-RACE was performed as previously described (Budak and Akpinar 2011; Kantar et al. 2010; Pandey et al. 2014) for nine targets. Four poly(A) mRNA pools were enriched from the WG total RNA samples of four durum varieties using the PolyATtract mRNA Isolation System III (Promega, USA). For each pool, 25 ng of poly(A) mRNA was directly ligated to a 44-nt 5′ RACE RNA adaptor (Electronic supplementary material Table S3) using T4 RNA ligase I (New England Biolabs, UK). Ligated RNA was used for cDNA synthesis with the SuperScript IV First-Strand Synthesis System (Invitrogen, USA) following the manufacturer's instructions. To amplify cleaved fragments of mRNA targets, a modified touch-down PCR was performed for each target using RNA adaptor-specific forward primers and gene-specific reverse primers (GSPs) (Electronic Supplementary Material Table S3). GSPs were designed using Primer3Plus (www.bioinformatics.nl/primer3plus) and the following criteria as previously described (Kantar et al. 2010), with slight adjustments: (1) GSPs were located at least 100–200 bp downstream of the miRNA/mRNA binding sites; (2) primer annealing temperatures were restricted to 65 ± 5 °C; (3) GC content of the primers was limited to 50 ± 10 %; and (4) the length of the primers were 20–26 nt.

Q3

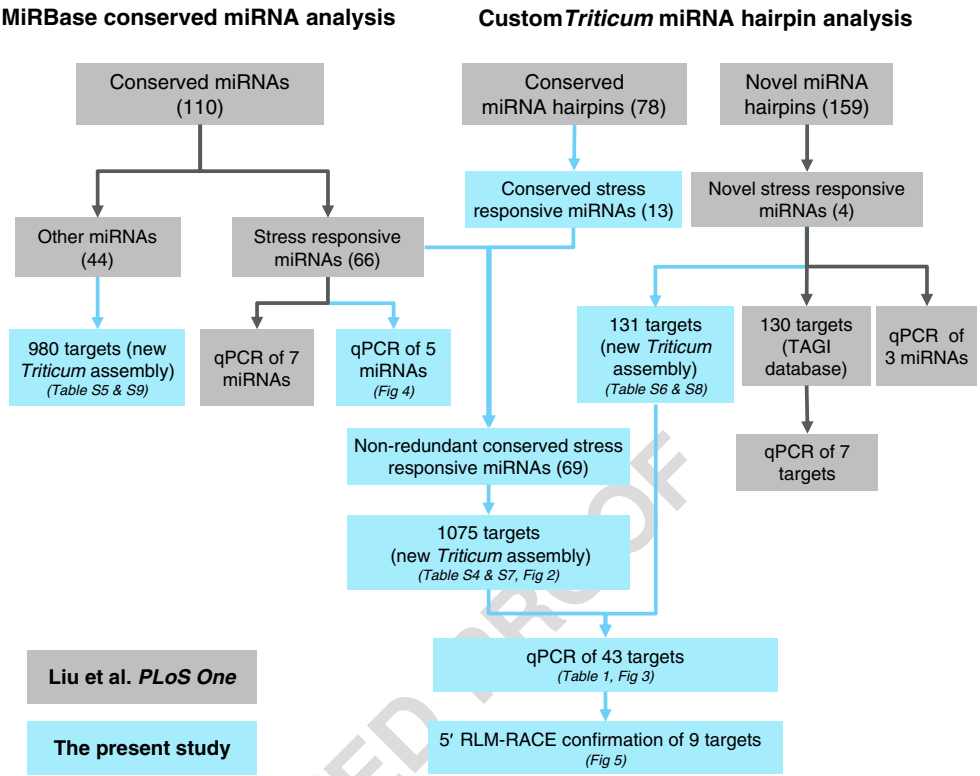


Fig. 1 A schematic flow chart of the research approach used in this study, continuing from previously published work. The *blue-coloured boxes* represent results from this study, while the *grey-coloured boxes* indicate results from a previous report (Liu et al. 2015b)

Response to Q3:
Yes all figures are correct.

The modified touch-down PCR conditions were: 94 °C for 2 min; followed by 5 cycles of 94 °C for 30 s, 70 °C for 30 s; followed by 5 cycles of 94 °C for 30 s, 68 °C for 30s, followed by 25 cycles of 94 °C for 30 s, 60–64 °C for 30 s and 70 °C for 30 s, followed by 72 °C for 10 min. Amplified PCR products of RACE fragments were extracted from a 3 % agarose gel for ideal separation with the PureLink Quick Gel Extraction Kit (Invitrogen, Australia). Purified RACE products were cloned with pGEM-T Easy vectors (Promega, USA). Individual positive clones were sequenced and the 5′ end sequence of the cleaved targets was obtained from at least six clones (Chen et al. 2016; Ding et al. 2014; Dong et al. 2013).

Results

Target prediction and GO analysis of all targets

To infer the biological functions of miRNA targets in durum wheat, psRNATarget program (http://plantgrn.noble.org/psRNATarget/) was employed with default settings for the in silico prediction of durum miRNA-targeted genes. An updated *Triticum* transcriptome assembly was used in this study as the reference dataset (Fig. 1). In total, 1075 targets were identified for 69 non-redundant conserved stress-responsive durum miRNAs (Electronic supplementary material Table S4), 980 targets for the other 44 conserved durum miRNAs (Electronic supplementary material Table S5) and 131 targets for 4 novel stress-responsive miRNAs (Electronic supplementary material Table S6). The psRNATarget results are in agreement with previous reports of miRNA target transcriptome in other crop species that a single miRNA may regulate multiple target genes and vice versa (Akpinar et al. 2015; Ferdous et al. 2016; Ma et al. 2015; Xie et al. 2014, 2015b). In durum, conserved Hvu-miR5049b, novel Ttu-miR008 and conserved Osa-miR1436 were predicted to have the most number of targets (99, 95 and 94 targets, respectively) (Electronic supplementary material Tables S5 and S6). No targets were found for Tae-miR1127, Bdi-miR159-5p, Bdi-miR5054, Gma-miR5368 and Gma-miR6300. The functions of some target genes were also not predicted, more than likely due to the incomplete annotation of the large and complex wheat genomes. Similar to previous reports in wheat (Alptekin and Budak 2016; Eren et al. 2015; Meng et al. 2013), the predominant post-transcriptional gene silencing mode for durum miRNAs appears to be mRNA cleavage as compared with translational inhibition (Electronic supplementary material Tables S4, S5 and S6). All putative targets were subjected to GO analysis to evaluate their potential functions (Electronic supplementary material Tables S7, S8 and S9). Target genes regulated by the conserved and novel stress-responsive durum miRNAs include transcription factors and gene families of various functions such as signal transduction, hormone responses, metabolic processes and cell development. The GO categorisation of conserved

Response to Q4: Table 1 needs to be corrected. Please see details below.

Q4 t1.1 **Table 1** List of 43 functional target candidates studied in four durum wheat genotypes

	Target no.	Target accession	Description	Length	miRNA name
t1.3	T1	CL1Contig1941	Heat shock protein 90	3269	Ath-miR396b[a]
t1.4	T2	CL33515Contig1	Protein phosphatase 2C 48	892	Tae-miR408
t1.5	*T3*	*CL33956Contig1*	*Auxin response factor 8-like*	*1051*	*Ath-miR160a[a]/ Sbi-miR160f[a]*
t1.6	*T4*	*CL3649Contig1*	*Auxin response factor 18-like*	*2459*	*Ath-miR160a[a]/ Sbi-miR160f[a]*
t1.7	T5	CL5358Contig1	L-ascorbate oxidase	1666	Ttu-miR008
t1.8	*T6*	*Contig00615a*	*Heat shock protein binding protein*	*2105*	*Tae-miR395b*
t1.9	T7	Contig00615b	Heat shock protein binding protein	2105	Hvu-miR5049c
t1.10	T8	Contig03837	Phytoene synthase 2—partial	1177	Ttu-miR008
t1.11	T9	Contig07291	Hypothetical protein TRIUR3_01074	1076	Cme-miR171f
t1.12	T10	Contig08755	Sucrose-phosphate synthase	3186	Zma-miR167a
t1.13	T11	Contig100623	Aberrant pollen transmission 1	3302	Bdi-miR827-3p[a]
t1.14	T12	Contig102950	Heat shock protein 90	2542	Ath-miR396b[a]
t1.15	T13	Contig104563	Cysteine-rich receptor-like protein kinase 26	2285	Ttu-miR008
t1.16	T14	Contig104812	Phytoene synthase 2	1323	Ttu-miR008
t1.17	T15	Contig112319	CBL-interacting protein kinase 24	2020	Bdi-miR171d
t1.18	T16	Contig112771	Disease resistance RPP8-like protein 3	1520	Osa-miR5071[b]
t1.19	T17	Contig113586	Cold shock-like protein	691	Ttu-miR008
t1.20	T18	Contig121164	Pin2-interacting protein X1	1616	Ath-miR396b[a]
t1.21	T19	Contig125505	Auxin response factor 9-like	2807	Bdi-miR397b
t1.22	*T20*	*Contig13056*	*Homeobox-leucine zipper protein HOX32*	*559*	*Ath-miR166b*
t1.23	*T21*	*Contig16465*	*Disease resistance protein RPM1*	*2377*	*Osa-miR5071[b]*
t1.24	T22	Contig24104	Serine threonine-protein kinase PBS1	1113	Hvu-miR5049d
t1.25	T23	Contig35578	Copper transporter	1990	Ttu-miR007
t1.26	*T24*	*Contig59374*	*Class III homeodomain leucine zipper protein*	*1261*	*Ath-miR166b*
t1.27	T25	Contig77300	Cell wall-associated hydrolase	2778	Tae-miR395b
t1.28	T26	gi|25156716|gb|CA601554.1|CA601554	Sucrose synthase 1	548	Zma-miR528a[b]
t1.29	*T27*	*gi|25242389|gb|CA663864.1|CA663864*	*Superoxide dismutase*	*542*	*Zma-miR398b*
t1.30	*T28*	*gi|32674180|gb|CD899852.1|CD899852*	*Heat shock protein 83*	*644*	*Osa-miR444b*
t1.31	T29	Isotig04129__gene=isogroup00173__length=3409__numContigs=7	Leucine-rich repeat receptor-like kinase	3409	Osa-miR393b
t1.32	T30	Isotig11160__gene=isogroup01194__length=864__numContigs=4	Two-component response regulator ARR3-like	864	Gma-miR164b
t1.33	T31	KukriC1047_2	Target of rapamycin isoform 1 (TOR1)	5809	Tae-miR395b
t1.34	*T32*	*KukriC12019_1*	*Disease resistance protein RPP13*	*1823*	*Osa-miR5071[b]*
t1.35	T33	KukriC13997_1	Disease resistance protein RPP13	1744	Bdi-miR7757
t1.36	T34	KukriC15_229	Disease resistance protein RGA2	650	Hvu-miR5049d
t1.37	T35	KukriC15_415	Heat shock protein 90	352	Ath-miR396b[a]
t1.38	T36	KukriC2179_19	Aberrant pollen transmission 1	948	Bdi-miR827-3p[a]
t1.39	T37	KukriC2179_6	Aberrant pollen transmission 1	3945	Bdi-miR827-3p[a]
t1.40	T38	KukriC460_3	NADH dehydrogenase	4612	Gma-miR408d[b]
t1.41	T39	KukriC7839_1	Abscisic stress ripening	818	Hvu-miR5049c

This transcript name needs to be black as others.

t1.42 **Table 1** (continued)

	Target no.	Target accession	Description	Length	miRNA name
t1.43	T40	KukriC8142_3	F-box protein At3g07870-like isoform X1	877	Bdi-miR167e[a]
t1.44	T41	KukriC827_4	Ubiquitin-conjugating enzyme E2 24	2690	Zma-miR399c
t1.45	T42	KukriC8474_2	3-oxo-delta(4,5)-steroid 5-beta-reductase-like	1109	Ttu-miR008
t1.46	T43	KukriC944_6	Auxin response factor 9-like	1576	Bdi-miR397b

This transcript name needs to be black as others.

Targets set in italics are validated with 5′RLM-RACE PCR (see Fig. 5). Contig00615a and contig00615b are the same gene transcript but with two different target regions for two different stress-responsive microRNAs. CL33956Contig1 (T3) and CL3649Contig1 (T4) are predicted to be targeted by both Ath-miR160a and Sbi-miR160f, where Ath-miR160a has a better expectation score in the psRNATarget prediction

Ath Arabidopsis thaliana, *Bdi Brachypodium distachyon*, *Hvu Hordeum vulgare*, *Gma Glycine max*, *Osa Oryza sativa*, *Sbi Sorghum bicolor*, *Tae Triticum aestivum*, *Ttu Triticum turgidum*, *Zma Zea mays*

[a] qPCR profile shown in Fig. 4

[b] qPCR profile previously published (Liu et al. 2015b)

Please could the superscript "a" and "b" be moved to the front of the miRNA name, thus avoiding any potential naming confusion.

stress-responsive durum miRNA-targeted transcripts is sorted by cellular components, molecular functions and biological processes (Fig. 2). According to the cellular components analysis, 1075 targets of conserved stress-responsive miRNAs are associated with 15 cell parts, with over half of them localised in the cytosol and the organelle membrane (Fig. 2a). Furthermore, GO categorisation revealed that these targets are associated with 22 different molecular functions, primarily participating in binding activities such as heterocyclic compound binding, organic cyclic compound binding, ion binding, small molecule binding and carbohydrate and derivative binding (Fig. 2b). In terms of biological processes, these putative targets are represented by 13 major categories, while metabolic processes and regulation could be classified into five and eight sub-categories respectively (Fig. 2c). The three most abundant biological processes are metabolic processes, regulation and response to stimuli, suggesting the extensive involvement of durum miRNAs in stress responses and gene regulation.

stress-sensitive

Expression profiles of 43 targets and five stress-responsive miRNAs

In order to confirm the differential gene expression of durum miRNAs and their putative targets when subject to water-deficit stress, qPCR profiling was carried out for 43 miRNA-targeted genes of interest (Table 1; Fig. 3) and five conserved stress-responsive durum miRNAs (Fig. 4) within two tissue types of four durum varieties. Overall, the targets exhibited stress-responsive, tissue-associated and genotype-dependent expression patterns (Fig. 3) as expected. For example, T30 (Two-component response regulator ARR3-like) was down-regulated in the flag leaf tissue under water-deficit stress in both stress sensitive varieties but up-regulated in the tolerant varieties. Similarly, T4 (CL3649Contig1, auxin response factor 18-like gene) and T11 (contig100623, Aberrant pollen transmission1) were down-regulated in both stress sensitive varieties but up-regulated in the stress-tolerant varieties in the developing head tissue. T7 (Heat shock binding protein gene) was only up-regulated under water-deficit stress in the flag leaf of all four durum varieties. T22 (Serine threonine-protein kinase PBS1), T36 and T37 (both Aberrant pollen transmission 1 genes) also exhibited a similar stress-responsive expression profile subject to tissue type, except for the developing head tissue of Tjilkuri. T19 (auxin response factor 9-like) was down-regulated in the developing head of all four varieties, but did not change in the flag leaf. T18 (Pin2-interacting protein X1) was up-regulated under stress in the flag leaf but down-regulated in the head of three varieties, with no significant changes in the variety Yawa (Fig. 3).

stress-sensitive

stress-sensitive

In terms of the expression profiles of the chosen miRNAs (Fig. 4), Ath-miR160a was down-regulated in both stress-tolerant varieties but slightly up-regulated or not changed in stress sensitive varieties in the flag leaf. Bdi-miR167e exhibited an opposite pattern in flag leaf samples where up-regulation was observed in the stress-tolerant varieties and down-regulation was observed in the stress-sensitive varieties. Ath-miR396b was up-regulated under water-deficit stress in the flag leaf of all durum varieties except Tjilkuri, but down-regulated in the head. Some negative correlation could be observed between miRNA-target pairs. For example, Ath-miR160a exhibited down-regulation in the flag leaf of Tamaroi and Yawa and up-regulation in Tjilkuri. Its targets T3 (auxin response factor 8-like) and T4 (auxin response factor 18-like) both exhibited inverted expression profiles (up-regulation in the flag leaf of Tamaroi and Yawa, down-regulation in Tjilkuri). However, such negative correlation was absent between Sbi-miR160f and T3/T4, indicating the possible predominant role of Ath-miR160a in the miRNA-target interaction.

For other miRNAs with multiple genes, the interaction between stress-responsive miRNA and individual targets could be different and quite complex, in some cases, subject to tissue

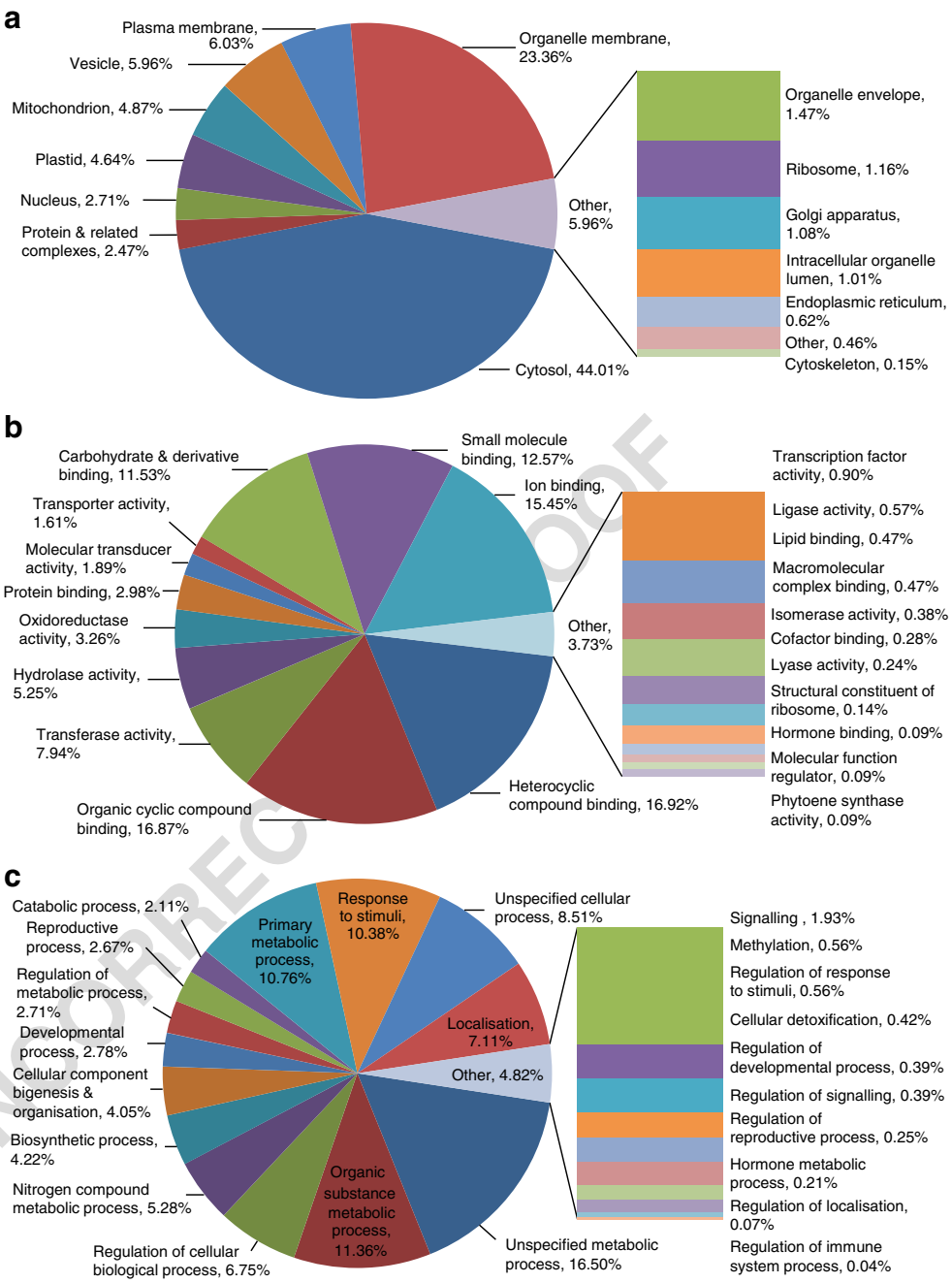
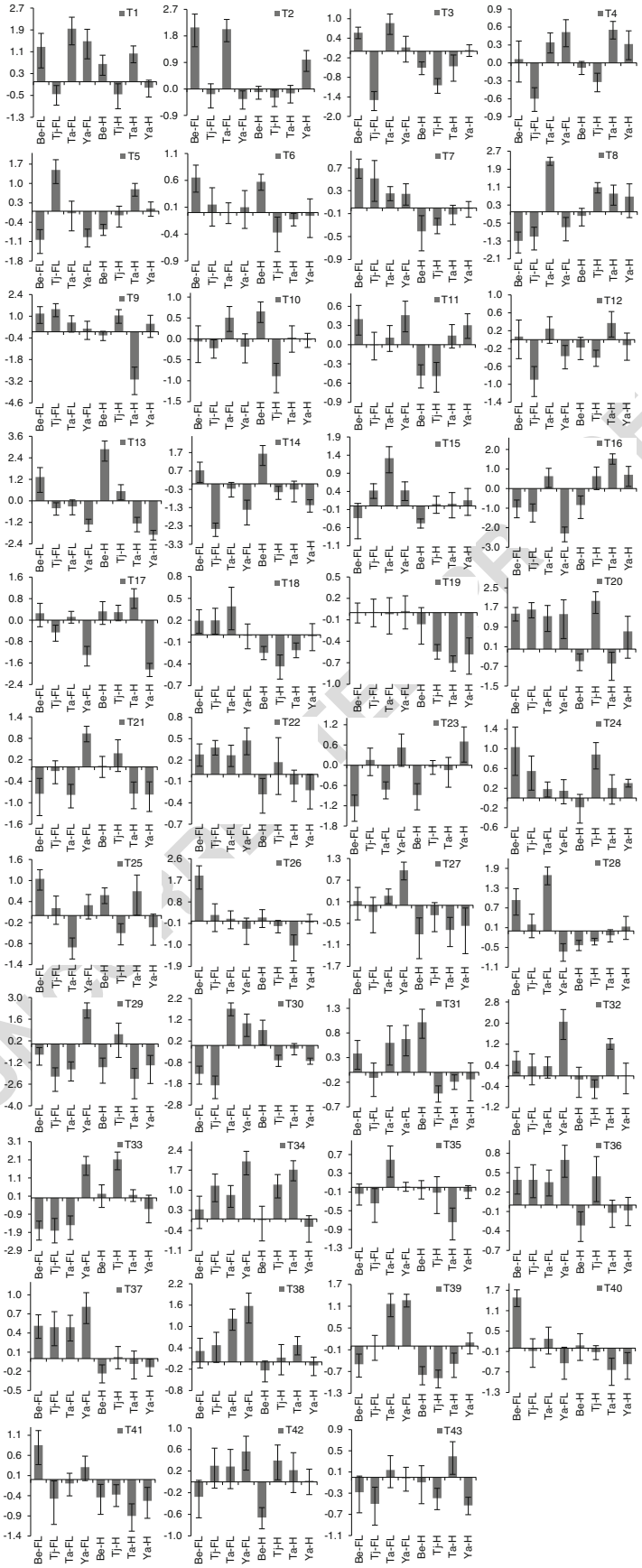


Fig. 2 Categorisation of 1075 predicted targets of 69 conserved stress-responsive miRNAs using Gene Ontology (GO) annotations. Pie charts representing different GO categories are based on the number of target sequences enriched in each category. GO terms at level 4 are used for **a** cellular component categorisation. GO terms at level 3 are used for **b** molecular function and **c** biological processes

type or genotype. For example, Ath-miR396b only exhibited negative correlation with its target T1 in the head of Tamaroi and EGA Bellaroi. However, for another target of Ath-miR396b, T35, such correlation could only be found in the flag leaf of EGA Bellaroi; while between Ath-miR396b and T18, no clear negative correlation could be observed in any of the genotypes. For target genes regulated by stress-responsive miRNAs quantified in our previous study (Liu et al. 2015b), their interaction is similarly complex. For instance, among T16, T21 and T32 (all targeted by Osa-miR5071), a negative correlation was only found in the head of EGA Bellaroi, Tamaroi and Yawa for T16; and in the head of Tamaroi and Tjilkuri for T32.

Cleavage sites during microRNA/mRNA binding

To validate the cleavage of target genes mediated by miRNAs, a modified 5′ RLM-RACE approach was performed as previously described (Budak and Akpinar 2011; Kantar et al. 2010; Pandey et al. 2014). The 5′ end fragments of nine miRNA-targeted genes were amplified using adaptor-specific universal forward primers and gene-specific reverse primers (Fig. 5). These nine validated targets include two auxin response factors (T3 and T4), a homeobox-leucine zipper protein HOX32 (T20), a class III homeodomain leucine zipper protein (T24), a heat shock protein 83 (T28), a heat shock protein binding



◀ **Fig. 3** Differentially expressed target genes in response to pre-anthesis water-deficit stress in two tissue types of four durum wheat genotypes revealed by qPCR. *FL* flag leaf, *H* developing head, *Ta* Tamaroi, *Ya* Yawa, *Be* EGA Bellaroi, *Tj* Tjilkuri, *Ath Arabidopsis thaliana*, *Bdi Brachypodium distachyon*, *Sbi Sorghum bicolor*. Log (2)-fold changes (mean ± SE) between control group (CG) and water-deficit stress group (WG) are shown for 43 target genes

protein (T6), a superoxide dismutase (T27) and two disease resistance proteins (T21 and T32). Similar to the studies conducted in other cereal crops (Budak and Akpinar 2011; Sun et al. 2014; Zhai et al. 2013), the majority of durum miRNAs regulate the expression of their protein-coding target genes by guiding the RISC to cleave the mRNA target predominantly after the 10th or the 11th position within the miRNA/mRNA binding region. However, the cleavage of the miR444b/T28 pair occurred after the 7th position.

Discussion

Water-deficit stress is one of the major abiotic stress factors significantly reducing durum wheat production in Mediterranean environments. To develop high-yielding elite crop varieties with improved stress tolerance, a thorough understanding of the complex molecular mechanisms underlying stress response and adaptation is crucial. The identification and manipulation of some abiotic stress-related or developmental-associated miRNA regulatory modules have facilitated the improvement of stress resistance and grain yield in other cereal species such as rice and bread wheat (Budak et al. 2015b; Feng et al. 2013; Gao et al. 2015; Qu et al. 2015; Yang et al. 2013; Zhang et al. 2013). The work presented here was based on the stress- and genotype-dependent changes, identified using next generation sequencing, in the miRNA transcriptome of durum undergoing pre-anthesis water-deficit stress (Liu et al. 2015b). Identification of the target genes regulated by these stress-responsive miRNAs is crucial to unravel their possible biological functions in adaptation of durum wheat to abiotic stress. Target genes can be predicted via bioinformatics tools that use the high sequence complementarity of plant mature miRNAs and their corresponding targets (Ku et al. 2015). The genome-wide in silico workflow using psRNATarget and Blast2GO software has become one of the most popular approaches for predicting and annotating putative miRNA-targeted transcripts with high confidence in crop species (Akpinar et al. 2015; Cheah et al. 2015; Ma et al. 2015; Pandey et al. 2014). However, caution must be taken when choosing the reference transcriptome dataset in cereals like durum wheat where the whole genome sequence is unavailable. Additionally, putative targets derived from this workflow need to be experimentally validated to confirm their actual response to abiotic stress. In this study, we therefore adopted a hybrid approach where *T. aestivum* and *T. turgidum* transcriptomes were assembled as the reference dataset in the computational psRNATarget-Blast2GO workflow, with targets of interest experimentally validated by qPCR and 5′ RLM-RACE. Using this approach, a total of 2186 target genes were predicted and annotated using previously identified durum miRNAs (Liu et al. 2015b) as queries. When comparing the computational target analysis of the four novel stress-responsive miRNAs, an increased number of annotated target genes were retrieved by using the new updated reference dataset as compared with the TAGI dataset used previously (Liu et al. 2015b). Quantitative PCR of 43 protein-coding targets and five miRNAs revealed their stress-responsive, tissue-associated and/or genotype-dependent expression profiles under pre-anthesis water-deficit stress. The interactions between miRNA-target pairs are quite complex based on their expression profiles. The negative correlation observed between stress-responsive durum miRNAs and their targets could be subject to tissue type and genotype in some cases, suggesting that the regulatory functions of certain miRNAs could be specific to certain tissue(s) and/or genotype(s). Where a miRNA is

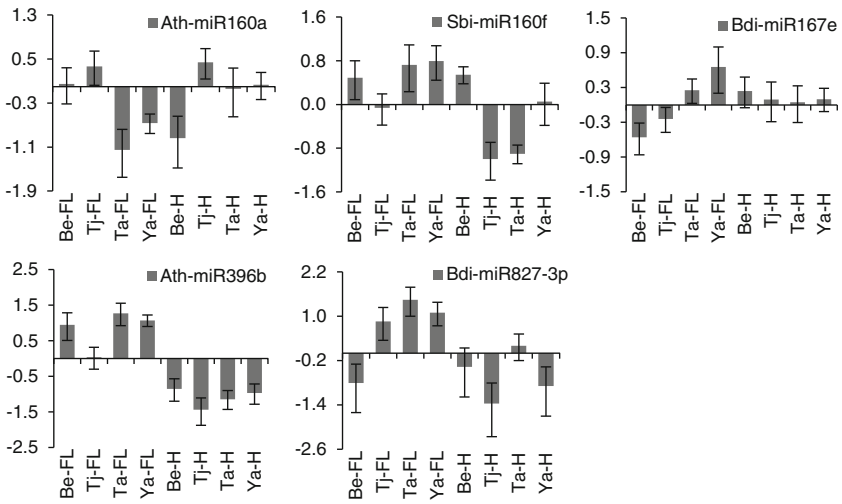


Fig. 4 Differentially expressed miRNAs in response to pre-anthesis water-deficit stress in two tissue types of four durum wheat genotypes revealed by qPCR. Log (2)-fold changes (mean ± SE) between control group (CG) and water-deficit stress group (WG) are shown for five microRNAs. These five microRNAs were randomly selected from the stress-responsive microRNAs that target the 43 functional target genes of interest selected in this study

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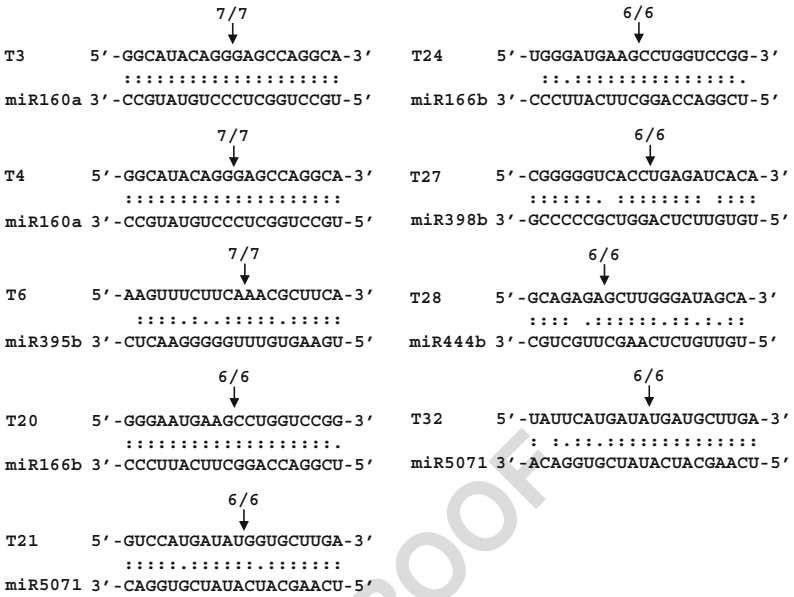

Fig. 5 Mapping of the mRNA cleavage sites induced by stress-responsive durum miRNAs using modified 5′ RLM-RACE. The targeted region of mRNA targets was aligned with the mature durum miRNA sequence. *Colons* indicate a Watson-Crick pairing; *dots* represent a mismatch. G-U wobbles are shown by *blanks*. The *arrows* indicate the 5′ termini of miRNA-guided cleavage products. The *numbers* indicate the frequency of the sequenced RACE products

targeting multiple genes (such as Ath-miR396b), the negative correlation only observed between certain targets and the miRNA suggests the possible specificity of miRNA silencing at a particular developmental stage or tissue during stress responses while some other targets remain unaffected. This highlights the complexity of miRNA regulatory networks during stress responses, where several factors such as miRNA regulatory interplay, other gene transcription events and target-binding capabilities could simultaneously affect the target mRNA abundance. 5′ RLM-RACE validation of nine targets of interest regulated by conserved stress-responsive durum miRNAs provides the first experimental evidence of protein-coding genes genuinely cleaved by miRNAs in durum wheat.

The two stress-tolerant varieties (Tamaroi and Yawa) and two stress sensitive varieties (EGA Bellaroi and Tjilkuri) used in this study were previously selected among an initial set of 20 durum genotypes evaluated for their performance under pre-anthesis water-deficit stress (Liu et al. 2015a). These four varieties exhibited significantly differential stress tolerance capacity in terms of their morphological and physiological traits and yield responses (Liu et al. 2015a) but relatively little is known about how this is achieved at the molecular level. The miRNA profiles of these closely related genotypes with contrasting stress sensitivities yielded striking differences, suggesting the importance of durum miRNAs in the stress tolerance mechanism (Liu et al. 2015b). The annotation of the targets of the stress-responsive durum miRNAs could therefore further our understanding of how responses to pre-anthesis water-deficit stress are regulated. These putative targets include many transcription factors and other functional proteins that participate in various physiological and biological processes, such as phytohormone signalling (including auxin response factors, protein phosphatase 2C and two-component response regulator ARR3-like proteins); metabolic processes (including sucrose-phosphate synthase, cell wall-associated hydrolase and phytoene synthases); osmoprotection activities (including nicotinamide adenine dinucleotide (NADH) dehydrogenase, superoxide dismutases, CBL-interacting protein kinases and serine-threonine kinases); developmental and reproductive events (including flowering locus T protein, squamosa promoter-binding-like proteins, aberrant pollen transmission 1 and NAC-domain containing proteins) and defence mechanisms (including heat shock proteins, cold shock proteins, disease resistance proteins and salt response proteins).

Several miRNA-target transcriptional factor regulatory circuits could be contributing to the genotypic water stress tolerance in durum through their involvement in hormone biosynthesis and signalling. Under stressful conditions, adjusted hormone signalling and biosynthesis are common survival strategies employed by crops to reallocate limited resources and energy to re-establish cellular homeostasis (Kohli et al. 2013; Peleg and Blumwald 2011). For example, auxin signalling is not only crucial for plant growth and development, but also is extensively involved in abiotic stress responses (Kohli et al. 2013; Sharma et al. 2015). In this study, two auxin response factor-like genes, ARF8 and ARF18, are validated as the targets of the conserved miR160a in durum wheat (Fig. 5). In the two stress-tolerant genotypes, ARF8 (T3) and ARF18 (T4) both showed up-regulation expression patterns in response to stress (Fig. 3), which is negatively correlated with the down-regulation of miR160a (Fig. 4). ARF8 is known to be the transcriptional activator of genes in the auxin responsive *GH3 (GRETCHEN HAGEN 3)* gene family, which encode auxin-conjugating proteins to control free cellular levels and therefore maintain auxin homeostasis (Ludwig-Müller 2011). While ARF18 acts as a positive

Please replace "alleviated" with "elevated" in lines 536 and 551

signalling regulator by suppressing a negative auxin signalling component, the *IAA16 (INDOLE ACETIC ACID-INDUCED PROTEIN 16)* gene (Oh et al. 2009). Alleviated ARF8 and ARF18 levels under stress could therefore lead to increased GH3 and reduced IAA16 proteins, contributing to enhanced auxin signalling and auxin homeostasis in the stress-tolerant genotypes. Coordinated and antagonistic ratios of auxin, which promotes cell division, and cytokinin, which promotes cell differentiation, could stimulate the development of root meristem and lateral root growth (Lavenus et al. 2013; Su et al. 2011), contributing to enhanced water and nutrient uptake in stressful environments.

Furthermore, as endogenous auxin can affect jasmonic acid biosynthesis, auxin response factors and GH proteins have also been demonstrated to promote reproduction through the modulation of jasmonic acid production (Liu et al. 2014; Tabata et al. 2010; Yadav et al. 2011; Zhang et al. 2015). Thus, alleviated ARF levels in the stress-tolerant durum genotypes could possibly contribute to the greater grain number observed in these varieties (Liu et al. 2015a). However, their specific functional roles require further investigation in durum floral organs and possible developing grains. Two ARF9-like genes targeted by Bdi-miR397b also exhibited differential stress-responsive expression patterns across four durum varieties. Moreover, stress-responsive miR393h is predicted to target two proteins in durum, which share similarity with the auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1) (KukriC1405_1 and KukriC3321_1). Given that TIR1 affects the abundance of ARFs (Sharma et al. 2015), the spatio-temporal expression of miRNAs fine-tuning auxin perception and signalling is likely to play a major role in the physiological responses of durum to pre-anthesis water-deficit stress. Interestingly, in other species, ARF6 and ARF8 are targeted by miR167 rather than miR160, whereas miR160 targets ARF10, ARF16 and ARF17 (Liu et al. 2014; Wang et al. 2005; Wu et al. 2006). This is possibly due to the species-specific evolution of miRNA regulatory circuits during the speciation process, suggesting that durum miR160 could possibly be an evolutionary functional synonym to miR167.

Some miRNA targets could also contribute to plant fitness under stressful conditions through direct regulation of hormone-associated genes involved in plant developmental events. Homeobox-leucine zipper protein HOX32 (T20) and Class III homeodomain-leucine zipper protein (T24) (both cleaved by miR166b), were generally up-regulated under water-deficit stress in the flag leaf tissue of all the durum varieties in this study. Homeodomain-leucine zipper (HD-Zip) transcription factors could participate in auxin-mediated plant development events as well as abiotic stress responses by directly regulating the expression of several genes associated with auxin biosynthesis, transport and signalling (Turchi et al. 2015). Specifically, Class III HD-Zip transcription factors could control apical embryo patterning, embryonic shoot meristem formation, leaf polarity, lateral organ initiation and vascular bundle development (Turchi et al. 2015). In Arabidopsis, miR166/165 controls root meristem size and growth through post-transcriptional regulation of the Class III HD-Zip proteins (Singh et al. 2014). Thus, the miR166/HD-Zip regulatory module might have the potential to be used for modulating root architecture to improve water and nutrient uptake.

Another target gene, T11, which is an Aberrant pollen transmission 1 (APT1), could be possibly contributing to reproductive development in durum under water-deficit stress. APT1 is a homologue of the SABRE and KIP (KINKY POLLEN) proteins which are required for cell elongation in root cortex and pollen tubes. Specifically, in maize and Arabidopsis, APT1 and KIP regulate secretory membrane trafficking, which is crucial to the high-demanding membrane vesicle accumulation at pollen tube tips (Procissi et al. 2003; Xu and Dooner 2006). In this study, T11 was up-regulated in the head of two stress-tolerant durum genotypes, suggesting its possible role in pollen development and reproduction in response to water-deficit stress. Target genes such as superoxide dismutase (SOD) could contribute to water stress tolerance through participating in antioxidant defence and osmoprotective systems. In this study, T27, a SOD was validated to be the target of miR398b. Plant miR398 and SODs is a well-studied regulatory module widely conserved across different plant species (Lu et al. 2011; Sunkar et al. 2006; Zhu et al. 2011). Under water-deficit stress, rapid accumulation of reactive oxygen species (ROS) results in cellular damage of biomolecules including DNA, proteins and lipids (Choudhury et al. 2013; Gill and Tuteja 2010). A BLAST search of T27 suggested that it is possibly a cytosolic CuZnSOD (data not shown). Cytosolic CuZnSODs play significant roles in ROS scavenging by catalysing cytotoxic ROS as antioxidant enzymes in cytosolic compartments during water-deficit stress responses (Faize et al. 2011). Increased cytosolic CuZnSOD activity under drought stress is related to the maintenance of the photosynthetic rate due to the positive effects of cytosolic ROS defence on the chloroplast, which is highly sensitive to extra-chloroplastic ROS damage (de Deus et al. 2015; Faize et al. 2011). In addition, transgenic plants overexpressing cytosolic CuZnSOD exhibited higher water use efficiency and photosynthetic activity, which all contributed to the increased tolerance against water-deficit stress (Faize et al. 2011; Lu et al. 2015). In this study, an increased level of SOD observed in the flag leaf of both stress-tolerant genotypes indicates the possible role of a durum miR398/SOD module in ROS detoxification and stress defence.

Another target quantified in this study, NADH dehydrogenase (T38) (putative target of miR408) could be contributing to water-deficit stress responses in durum through adjusted respiration activities. Mitochondrial respiration in plants provides energy for cellular biosynthesis through oxidative

"and -sensitive" in lines 690 and 698

phosphorylation of the respiratory substrates and molecular oxygen produced from active photosynthesis (Millar et al. 2011). Mitochondrial NADH dehydrogenase Complex I is of great importance to the electron transport chain in the classical respiratory pathway and to ATP production that is needed for cell maintenance and growth (Fromm et al. 2016). NADH dehydrogenase could also participate in the alternative non-phosphorylating respiratory pathway which alters ATP biosynthesis efficiency and is associated with cellular oxidative stress response due to the reduction of ROS production by the classical electron transport chain (Millar et al. 2011). Pastore et al. has demonstrated that durum wheat mitochondria can diminish ROS generation through three energy-dissipating systems and play a central role in cell adaptation to drought stress (Pastore et al. 2007). Under water-deficit stress, NADH dehydrogenase was up-regulated in the flag leaf tissue of all four durum varieties, but to a greater extent in the stress-tolerant varieties. The active spatial-temporal regulation of respiratory pathways involving NADH dehydrogenase, together with genotypic responses of cytosolic SOD activity, could possibly form a mitochondrion-cytosol-chloroplast circuit regulating cellular redox homeostasis in the leaves, ultimately contributing to water-deficit stress tolerance in durum wheat.

Other targets validated in this study are proteins involved in the plant defence system, including heat shock protein 83 (T28) (targeted by miR444b), heat shock protein 90 (T35) (targeted by miR396b), heat shock protein binding protein (T6) (targeted by miR395b) and two disease resistance proteins (T21, T32) targeted by miR5071. Heat-shock proteins are molecular chaperones that facilitate protein refolding, protein stabilisation, membrane assembly and protein import and translocation under stressful conditions (Santhanagopalan et al. 2015; Wang et al. 2004; Xu et al. 2013). Heat shock protein 90-based chaperone machinery also participates in signal transduction and may affect the synthesis of proline, an osmoprotectant, under abiotic stresses (Xu et al. 2013). Thus, these miRNA-targeted heat shock proteins might be playing an important role in protecting durum plants against water-deficit stress by preventing protein aggregation, maintaining protein conformation and re-establishing cellular homeostasis. Interestingly, heat shock protein 90 could also associate with and modulate disease resistance protein RPM1 (a validated target of miR5071) in Arabidopsis (Hubert et al. 2003). However, elucidation of the functional roles of heat shock proteins and disease-resistance proteins in durum water stress response requires future investigation.

Conclusions

To exploit the genetic resources for the development of stress-tolerant crops, a thorough understanding of the complex stress response and adaptation mechanisms at the molecular level is of great importance. Studying stress-responsive miRNA-target regulatory modules in different stress-tolerant and sensitive crop varieties provides insight in the transcriptional and post-transcriptional aspects of the stress response molecular networks governed by small RNAs. Reported here is a comprehensive prediction of the miRNA target transcriptome for durum wheat, and a comparative experimental investigation of miRNAs and target genes of interest in the context of their stress-responsive behaviours in the contrasting water stress-tolerant and sensitive durum genotypes. Among the 1075 and 131 putative target genes identified for 69 conserved and four novel stress-responsive miRNAs, a significant number of target transcripts were annotated to be transcription factors and functional proteins that are extensively involved in water-deficit stress response and plant development. The stress-responsive and genotype-dependent expression patterns of miRNAs and functional target genes suggest the involvement of miRNA-target regulatory modules in different abiotic stress defence pathways. Specifically, genes (such as auxin-response factors, HD-Zip proteins, SOD and heat shock proteins) associated with ABA response, auxin signalling, ROS scavenging, osmoprotection and lateral organ development were experimentally validated to be the genuine targets of stress-responsive miRNAs in durum wheat for the first time. Given the functional importance of these miRNAs and their targets in water-deficit stress adaptation, they have potential to be incorporated into strategies (such as RNAi technology) for improving stress tolerance in durum wheat. Target genes with negative roles in stress defence could be suppressed with induced expression of their corresponding stress-responsive miRNAs, which could effectively 'switch-off' the unfavourable pathways during stress. On the other hand, the expression of target genes acting as positive regulators of stress could be enhanced with the suppression of the miRNAs, therefore contributing to the accumulation of beneficial target products for the plant to cope with stress. Results derived from this study could support future research on the characterisation of individual miRNA regulatory modules and their specific biological functions. While the complete genome sequence for durum may be in its formative years, having this information in the future will further assist the endeavour to fully understand this crop's miRNA repertoire and their functions in stress response, especially the species-specific novel miRNA-mediated regulatory pathways.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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In line 770, replace "." with "," after the word "progenitor"; also italics for genus species name.

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Chapter 5 Addendum

Supplementary materials available online via DOI

Electronic supplementary materials Table S1. qPCR primers of 43 target genes used in this study.

*Please click on the following DOI link or scan the QR code to download this supplementary material. The size of this table is not suitable for thesis binding.

http://dx.doi.org/10.1007/s10142-016-0515-y

Electronic supplementary materials Table S2. Forward qPCR primers of five stress-responsive durum miRNAs used in this study.

Forward miRNA-specific primers were designed based on the full mature miRNA sequences. The universal adaptor-specific reverse primer was provided in the NCode VILO miRNA cDNA synthesis kit (primer sequence not provided by the manufacturer).

Mature miRNA sequence	**miRNA**	**Source**	**Primer (5' to 3')**
TGCCTGGCTCCCTGTATGCCA	MIR160a	*Arabidopsis thaliana*	CTGGCTCCCTGTATGCCAAA
TGCCTGGCTCCCTGAATGCCA	MIR160f	*Sorghum bicolor*	GGCTCCCTGAATGCCAAAA
AGGTCATGCTGGAGTTTCATC	MIR167e	*Brachypodium distachyon*	AGGTCATGCTGGAGTTTCATCAA
TTCCACAGCTTTCTTGAACTT	MIR396b	*Arabidopsis thaliana*	CCTTCCACAGCTTTCTTGAACTT
TTAGATGACCATCAGCAAACA	MIR827-3p	*Brachypodium distachyon*	GTTAGATGACCATCAGCAAACAAAA

Electronic supplementary materials Table S3. 5′ RLM-RACE adaptor and primers used in this study.

*Please click on the following DOI link or scan the QR code to download this supplementary material. The size of this table is not suitable for thesis binding.

http://dx.doi.org/10.1007/s10142-016-0515-y

Electronic supplementary materials Table S4. Predicted target genes of 69 conserved water-deficit stress-responsive miRNAs and their GO annotations.

*Please click on the following DOI link or scan the QR code to download this supplementary material. The size of this table is not suitable for thesis binding.

http://dx.doi.org/10.1007/s10142-016-0515-y

Electronic supplementary materials Table S5. Predicted targets of 44 conserved durum miRNAs (identified using MiRBase) and their GO analysis results.

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http://dx.doi.org/10.1007/s10142-016-0515-y

Electronic supplementary materials Table S6. Predicted targets of four novel stress-responsive durum miRNAs identified using the new *Triticum* assembly and their GO analysis results.

*Please click on the following DOI link or scan the QR code to download this supplementary material. The size of this table is not suitable for thesis binding.

http://dx.doi.org/10.1007/s10142-016-0515-y

Electronic supplementary materials Table S7. Combined Gene Ontology classification at different GO levels of the predicted targets of 69 conserved stress-responsive miRNAs for biological processes (**a**), molecular functions (**b**) and cell components (**c**).

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http://dx.doi.org/10.1007/s10142-016-0515-y

Electronic supplementary materials Table S8. Combined Gene Ontology classification at different GO levels of predicted targets of four novel stress-responsive miRNAs for biological processes (**a**), molecular functions (**b**) and cell components (**c**).

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Electronic supplementary materials Table S9. Combined Gene Ontology classification at different GO levels of predicted targets of 44 conserved durum miRNAs for biological processes (**a**), molecular functions (**b**) and cell components (**c**).

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Chapter 6

Statement of Authorship

Title of Paper	Genotypic water-deficit stress responses in durum wheat: association between physiological traits, microRNA regulatory modules and yield components
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Principal Author

Name of Principal Author (Candidate)	Haipei Liu		
Contribution to the Paper	Designed the experiments, conducted the research, analysed the data and drafted the manuscript.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	22/08/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Amanda Able		
Contribution to the Paper	Designed the experiments, analysed the data and drafted the manuscript.		
Signature		Date	22/08/16

Name of Co-Author	Jason Able		
Contribution to the Paper	Designed the experiments, analysed the data, drafted the manuscript and acted as the corresponding author.		
Signature		Date	22/08/16

FUNCTIONAL PLANT BIOLOGY

Genotypic water-deficit stress responses in durum wheat: association between physiological traits, microRNA regulatory modules and yield components

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Keyword:	Triticum spp., Abiotic stress, Gene regulation, Plant response

Genotypic water-deficit stress responses in durum wheat: association between physiological traits, microRNA regulatory modules and yield components

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Abridged title

Genotypic water-deficit stress responses in durum wheat

Summary Text for the Table of Contents

Pre-anthesis water-deficit stress causes detrimental effects on the production of crops such as durum wheat in rain-fed areas. In stress tolerant varieties, the regulation of microRNA160 and the mRNA that it targets, auxin response factors, are potentially associated with the unaffected leaf relative water content and chlorophyll content, and the coordinated control of stomatal aperture, which ultimately contribute to the maintenance of grain number and yield. Together, these findings suggest the importance of durum microRNA regulatory modules in water stress responses and provide useful information for improving stress tolerance in breeding.

Abstract

In Mediterranean environments, water-deficit stress that occurs prior to anthesis significantly limits durum wheat (*Triticum turgidum* L. ssp. *durum*) production. Stress tolerant and sensitive durum varieties exhibit genotypic differences in their response to pre-anthesis water-deficit stress as reflected by yield performance, but our knowledge of the mechanisms underlying tolerance is limited. We have previously identified stress responsive durum microRNAs (miRNAs) which could contribute to water-deficit stress tolerance by mediating post-transcriptional silencing of genes that lead to stress adaptation [e.g. miR160 and its targets *ARF8* (*auxin response factor 8*) and *ARF18*]. However, the temporal regulation pattern of miR160-*ARF*s after induction of pre-anthesis water-deficit stress in sensitive and tolerant varieties remains unknown. Here, the physiological responses of four durum genotypes were described by chlorophyll content, leaf relative water content, and stomatal conductance at seven time-points during water-deficit stress from booting to anthesis. qPCR examination of miR160, *ARF8* and *ARF18* at these time-points revealed a complex stress-responsive regulatory pattern, in the flag leaf and the head, subject to genotype. Harvest components and morphological traits measured at maturity confirmed the stress tolerance level of these four varieties for agronomic performance, and their potential association with the physiological responses. In general, the distinct regulatory pattern of miR160-*ARF*s among stress tolerant and sensitive durum varieties suggests that miRNA-mediated molecular pathways may contribute to the genotypic differences in the physiological traits, ultimately affecting yield components (e.g. the maintenance of harvest index and grain number).

Keywords: water-deficit stress; physiological traits; microRNA; auxin response factors; durum

Introduction

Durum wheat (*Triticum turgidum* L. ssp. *durum*, AABB, 2n = 4x = 28) is a major cereal crop mostly grown under rain-fed conditions in the Mediterranean region. With natural water availability for agricultural production becoming more limiting, growing emphasis has been placed on the understanding of water stress response mechanisms that could be exploited for crop improvement. In Australia, most durum growing regions are characterised by fluctuating and insufficient seasonal precipitation, which leads to the occurrence of moderate water-deficit stress prior to the anthesis stage which may intensify during grain filling (French and Schultz 1984; Nicholls *et al.* 1997; Garcia del Moral *et al.* 2003). For cereal crops, pre-anthesis water deficiency mainly affects the final grain yield via grain number reduction per plant, possibly due to a higher rate of spikelet abortion as well as pollen sterility (Praba *et al.* 2009; Sanjari Pireivatlou and Yazdansepas 2010). Specifically for durum wheat, limited studies have been conducted to characterise the effects of pre-anthesis water stress, despite the significant effects it could have on crop yield. Our previous study determined the genotype-dependent responses of 20 durum wheat varieties and breeding lines to pre-anthesis water-deficit stress (starting at the booting stage) by describing their physiological performance at anthesis (15 days after booting), and the final harvest components and morphological traits at maturity (Liu *et al.* 2015a). In general, stress tolerant durum genotypes exhibited adaptive physiological and morphological responses that enabled the plant to endure stressful conditions and achieve reproductive success (i.e. the maintenance of grain number and less yield loss), when compared to stress sensitive genotypes (Liu *et al.* 2015a). However, no study to date has reported on the temporal analysis of either physiological or molecular responses to water-deficit stress in durum wheat from booting to flowering.

On the molecular level, the regulatory roles of microRNAs (miRNAs, a type of small non-coding RNAs) in abiotic stress responses and plant development (especially reproductive processes) have been demonstrated to be crucial to plant fitness and crop production, which could be exploited to develop high-yielding stress tolerant varieties, achieving SMARTER cereal breeding (as reviewed by Liu *et al.* 2016a). miRNAs mainly modulate post-transcriptional silencing and translational repression of target genes that encode transcription factors and key proteins involved in signal transduction pathways, affecting almost all aspects of plant development and fitness, such as vegetative branching, leaf morphology, flowering, and reproductive organ development (Liu *et al.* 2016a). In our previous studies (Liu *et al.*

2015b; Liu *et al.* 2016b), the miRNA transcriptome of water stress tolerant and sensitive durum varieties exhibited genotypic regulation patterns at anthesis in response to water-deficit stress that started at booting. Expression profiling of target genes of the previously identified stress responsive durum miRNAs revealed that two contigs encoding auxin response factors (ARFs) were upregulated in the flag leaf of stress tolerant genotypes but downregulated in the stress sensitive genotypes (Liu *et al.* 2016b). The phytohormone auxin has been well-known to regulate a wide range of biological processes involved in plant development and responses to abiotic factors including water deficit (Ludwig-Müller 2011; Sharma *et al.* 2015) by upregulating auxin-responsive genes that are also involved in stress adaptation (Jain and Khurana 2009). The promoters of auxin-responsive genes have conserved elements such as AuxRE (auxin response element, TGTCTC) (Hagen and Guilfoyle 2002; Guilfoyle and Hagen 2007), to which ARFs could specifically bind to regulate their gene expression on the transcriptional level (Guilfoyle and Hagen 2007). The link between auxin signalling and miRNA-mediated stress response pathways may be explained because miRNAs targeting *ARF*s are responsive to various abiotic stressors. In arabidopsis (*Arabidopsis thaliana*) and several other species, *ARF6* and *8* are targeted by miR167 (Wu *et al.* 2006; Liu *et al.* 2014), while *ARF10*, *ARF16* and *ARF17* are the targets of miR160 (Mallory *et al.* 2005; Wang *et al.* 2005). Specifically in durum wheat, our previous report validated that *ARF8* and *ARF18*, are targeted by miR160 (Liu *et al.* 2016b). miR160 has been reported to be water-deficit stress responsive in several cereal species including durum wheat (Liu *et al.* 2016a). Due to the multiple functions that ARFs play in diverse biological processes, the expression pattern of the miR160-*ARF*s module at different stages of water-deficit stress could therefore possibly explain the differences in physiological performance among stress tolerant and sensitive durum genotypes.

In this study, two stress tolerant and sensitive Australian durum wheat varieties were characterised for their genotypic responses to pre-anthesis water-deficit stress at the physiological and molecular level. Physiological traits including chlorophyll content, leaf relative water content, and stomatal conductance measured at seven time-points after stress treatment from booting to anthesis exhibited differential responses between stress tolerant and sensitive durum varieties, as well as their yield components and morphological traits (plant height, fertile tiller number and main spike length) measured at harvest. Distinct expression profiles of miR160, *ARF8*, and *ARF18* characterised by temporal qPCR analysis

in the flag leaf and the developing head indicate the possible regulatory roles of miR160-*ARF*s in the pre-anthesis stress response mechanisms.

Methods

Plant materials, water-deficit stress treatment and sampling

For the four durum wheat varieties used in this study, Tamaroi and Yawa are water-deficit stress tolerant genotypes; while EGA Bellaroi and Tjilkuri are water-deficit stress sensitive (Liu *et al.* 2015a). Durum seeds were provided by Durum Breeding Australia's (DBA) southern node breeding program (The University of Adelaide). Plants were grown as previously described (Liu *et al.* 2015a) under glasshouse conditions at 22°C/16°C day/night temperature with a 12 h photoperiod, 45% relative humidity. Briefly, all plants were well-watered to field capacity [12% soil water content (SWC)] from germination to booting stage. From booting, SWC of the water-deficit stress group (WG) of each genotype was maintained at 6% until harvest, while the control group (CG) continued to be well-watered (SWC maintained at 12%) (Liu *et al.* 2015a). For each genotype, both flag leaf and the developing head on the main stem were sampled at different time-points after treatment [0, 3, 6, 9, 12, 15 and 18 DAT (days after treatment)]. For each sampling point, three flag leaf samples and three head samples were taken from three individual biological replicates. A total of 156 flag leaf samples [84 CG samples: four genotypes × seven sampling points (0 to 18 DAT) × three biological replicates; 72 WG samples: four genotypes × six sampling points (3 to 18 DAT) × three biological replicates)] and 156 developing head samples were collected and frozen immediately in liquid nitrogen, and stored at -80°C for further use.

Measurement of physiological, morphological traits and yield components

Chlorophyll content, leaf relative water content (RWC), and stomatal conductance were measured at noon (6 h of the 12 h photoperiod) at different time-points of stress (0, 3, 6, 9, 12, 15, 18 DAT) on the main stem of four biological replicates. Measurements of chlorophyll content were made five times along the middle section of the flag leaf with a chlorophyll meter (SPAD-502; Konica Minolta, Osaka) for each plant, and the mean value listed as SPAD units was used for analysis. RWC was measured on the penultimate leaf (Liu *et al.* 2015a). Fresh leaves were sampled and weighed immediately to record fresh weight (FW).

Leaves were then placed in distilled water for 5 h in the dark and weighed again to record turgid weight (TW). Dry weight (DW) was recorded after oven drying at 70°C for 24 h. RWC (%) was estimated using the formula: (FW – DW)/(TW – DW) × 100 (Barrs and Weatherley 1962). Stomatal conductance was measured on both the abaxial and adaxial surfaces along the middle section of the flag leaf, using a Delta-T AP4 porometer (Delta-T Devices Ltd, UK).

Upon maturity, durum plants were harvested to measure grain weight per plant, number of grains per plant, biomass, plant height, number of fertile tillers per plant, and main spike length (Liu *et al.* 2015a) with four biological replicates in both the CG and WG for each variety. Plant height was obtained by measuring from the base of the stem to the tip of the spike (main stem, awns not included). Main spike length was measured on the main stem from the base of the first spikelet to the tip of the last spikelet (awns not included). Harvest index was calculated as the ratio of grain dry weight to biomass (Donald 1962).

Total RNA extraction and qPCR profiling of miR160a/ARFs

A total of 312 total RNA samples (from the 156 flag leaf samples and 156 developing head samples) were isolated with Tri reagent (Sigma-Aldrich, Australia) following the manufacturer's instructions. The concentration and quality of total RNA samples were measured by spectrophotometric analysis at 260 nm and 280 nm using a NanoDrop Lite spectrophotometer (Thermo Scientific, USA). High quality RNA, as assessed by electrophoresis on a 2% agarose gel, was used for cDNA synthesis and subsequent qPCR analysis. A total of 312 poly(A)-tailed cDNA samples (156 flag leaf samples and 156 developing head samples) were synthesised using the MystiCq microRNA cDNA Synthesis Mix Kit (Sigma-Aldrich, Australia) according to manufacturer's instructions. Expression profiles of durum wheat miR160 and its validated targets, *ARF8* and *ARF18*, were quantified using SYBR Green reagent (iQ TM supermix, BioRad, USA) on a ViiA™ 7 Real-Time PCR machine (Applied Biosystems, USA). For the amplification of *ARF8* and *ARF18*, forward and reverse primers were designed to include the miRNA/target binding region in qPCR products, ensuring the quantification of uncleaved target transcripts (see Table S1, available as Supplementary Material to this paper) (Liu *et al.* 2016b). For the amplification of miR160, a forward miRNA-specific primer was designed based on the full mature miRNA sequence and the universal adaptor-specific reverse primer was provided in the MystiCq microRNA

cDNA Synthesis Mix Kit. Melting curves were performed and evaluated at the end of each qPCR reaction to ensure specificity. The comparative CT ($^{\Delta\Delta}$CT) method was used to calculate the relative expression of miR160 and the *ARF*s with GAPDH as the reference gene for its stable expression across durum wheat samples under water-deficit stress (Liu *et al.* 2015b; Liu *et al.* 2016b).

Statistical analysis

Statistical analysis of glasshouse data was performed as described previously (Liu *et al.* 2015a). Briefly, student's *t*-tests were performed to detect the significant changes in physiological traits, morphological traits and yield components in response to water-deficit stress for each genotype using GENSTAT 15th edition (VSN International Ltd, Hemel Hempstead, UK). Where appropriate, a *P* value of 0.05 was used to determine significance. Correlation coefficients were also calculated for all yield-component combinations. Correlation coefficients of the physiological parameters at 15 DAT were calculated separately for stress tolerant and sensitive varieties. For the qPCR expression analysis, log (2)-fold changes (mean ± SE) between the WG and CG at different time-points of stress were calculated for each genotype (Liu *et al.* 2016b).

Results

Stress tolerant and sensitive varieties exhibited differential physiological responses to water-deficit stress from booting to flowering

For all four genotypes, their chlorophyll content in CG plants slightly increased from booting (0 DAT) to around anthesis stage (18 DAT) (Fig. 1). At different time-points of water-deficit stress, the chlorophyll content of two stress tolerant genotypes (Tamaroi and Yawa) was lower in the WG compared to the CG, although this was not significant ($P > 0.05$). However, for the two stress sensitive genotypes, the chlorophyll content of the WG plants was significantly lower than the CG ($P < 0.05$) at all time-points for EGA Bellaroi and at 9 DAT to 18 DAT for Tjilkuri.

For leaf relative water content, its value in the CG of all four genotypes is similar (ranging from 94% to 98%) from booting to anthesis (Fig. 2). For stress tolerant genotypes, the RWC appears to be lower in the WG when compared with CG, but no significant

206 difference was detected (except for 18 DAT in Tamaroi). By 18 DAT, the average RWC in
207 the WG of Tamaroi was 91.7% (compared with 97.2% in the CG), while the average RWC in
208 the WG of Yawa was 93.6% (compared with 95.1% in the CG). However, a significant
209 reduction of RWC ($P < 0.05$) between WG and CG plants was observed in both EGA
210 Bellaroi and Tjilkuri at 6 to 18 DAT, with an even higher reduction in Tjilkuri. At 18 DAT,
211 the RWC of the stressed EGA Bellaroi plants dropped to 82.5% (12.7% lower than the CG).
212 For Tjilkuri, the RWC of the WG treatment was 75.0% (19.6% lower than CG).

213 Comparisons of stomatal conductance between the control and stress treatments in the
214 four durum varieties were made on both adaxial (Fig. 3*a*) and abaxial surfaces (Fig. 3*b*).
215 Overall, stomatal conductance on the abaxial surface of the flag leaf appeared to be more
216 sensitive to stress than the adaxial surface, regardless of genotype. In addition, the two stress
217 tolerant genotypes (Tamaroi and Yawa) showed less reduction in stomatal conductance on
218 both abaxial and adaxial surfaces, compared with the two stress sensitive genotypes (EGA
219 Bellaroi and Tjilkuri). Specifically, at 18 DAT, the adaxial stomatal conductance of the two
220 stress tolerant genotypes, Tamaroi and Yawa, was 68.3% and 64.4% lower in the WG
221 treatment than the controls respectively. However, the adaxial stomatal conductance at 18
222 DAT was 80.2% and 89.0% lower in the WG treatment than the control for the stress
223 sensitive genotypes EGA Bellaroi and Tjilkuri respectively. A similar pattern was observed
224 for stomatal conductance on the abaxial surface at 18 DAT with a smaller reduction in the
225 two tolerant genotypes in the WG treatment (86.8% and 81.1% lower than the control,
226 Tamaroi and Yawa respectively) compared with the two sensitive genotypes (93.9% and
227 96.7% lower than the control, EGA Bellaroi and Tjilkuri respectively). Interestingly, for both
228 abaxial and adaxial surfaces of all four genotypes, the steepest decline in stomatal
229 conductance was observed at the start of the water-deficit stress treatment (3 DAT). From this
230 point onwards, for the two stress tolerant genotypes, their WG stomatal conductance
231 remained stable as the plant developed to flowering under stress. For example, the adaxial
232 and abaxial stomatal conductance of Tamaroi stressed plants at 3 DAT was 187.0 and 34.5
233 mmol m^{-2} s^{-1}, while at 18 DAT the values were 296.3 and 59.8 mmol m^{-2} s^{-1} respectively.
234 However for the stress sensitive varieties, their disrupted stomatal conductance in the WG
235 treatment continued to decrease, reaching almost complete stomatal closure especially on the
236 abaxial side at 18 DAT. For instance, the adaxial and abaxial stomatal conductance of
237 Tjilkuri at 3 DAT was 97.3 and 22.2 mmol m^{-2} s^{-1} but at 18 DAT the values were 58.8 and
238 10.5 mmol m^{-2} s^{-1} respectively.

Correlation coefficients of the studied physiological traits were calculated at 15 DAT for stress tolerant and sensitive varieties separately to evaluate the possible links between physiological responses at flowering (Table 1). Stronger correlations were observed among the physiological traits measured in the stress sensitive varieties, EGA Bellaroi and Tjilkuri. Leaf relative water content is positively correlated with the stomatal conductance on the adaxial surface (r = 0.87) and the abaxial surface (r = 0.77). The correlation between chlorophyll content and the stomatal conductance is relatively strong (r = 0.66 for the adaxial surface and r = 0.73 for the abaxial surface) while the correlation between chlorophyll content and leaf relative water content is moderate (r = 0.50).

Stress tolerant varieties had less reduction in harvest components and morphological traits upon maturity

Overall, for all four durum wheat varieties, the biomass, grain weight, and grain number per plant were reduced under water-deficit stress compared with the controls (Table 2). The reduction in biomass was significant for both stress tolerant and sensitive varieties ($P < 0.05$). However, significant reductions of grain weight and grain number per plant due to stress was only observed for the two stress sensitive varieties, EGA Bellaroi and Tjilkuri. A significant reduction in the harvest index was also only observed in the two stress sensitive genotypes ($P < 0.05$), while this trait was maintained in the tolerant genotypes.

Plant height and fertile tiller number per plant were generally reduced under water-deficit stress compared with the control treatment (Table 2). Significant reductions ($P < 0.05$) in both of these traits were observed only in the two stress sensitive genotypes (EGA Bellaroi and Tjilkuri). For main spike length, no significant difference ($P > 0.05$) was found for any genotype between the CG and WG treatments. However, the two stress tolerant genotypes tended to have longer main spikes under water limiting conditions while EGA Bellaroi and Tjilkuri tended to show a reduced main spike length. Of the harvest components evaluated, grain weight had strong positive correlations with biomass (r = 0.93), grain number (r = 0.97) and harvest index (r = 0.95) (see Table S2, available as Supplementary Material to this paper). Grain number also exhibited a strong positive correlation with harvest index (r = 0.93). Of the harvest components and morphological traits evaluated, fertile tiller number had a strong positive correlation with grain weight (r = 0.82), grain number (r = 0.89) and harvest

index (r = 0.83). Plant height exhibited moderate positive correlations with biomass (r = 0.73), grain weight (r = 0.74) and grain number (r = 0.68).

The miR160-ARFs module exhibited genotypic regulatory patterns at different time-points of water-deficit stress

To characterise the gene expression profile of the miR160-*ARF*s regulatory module under water-deficit stress treatment between booting and flowering, qPCR profiling was carried out for *ARF8*, *ARF18* and miR160 at different time-points of stress within two tissue types of four durum varieties. Overall, the stress-responsive expression patterns of miR160, *ARF8* and *ARF18* differed across genotypes and tissue types.

The expression profile of *ARF8* exhibited a general inverted regulatory pattern between stress tolerant varieties (Tamaroi and Yawa) and sensitive varieties (EGA Bellaroi and Tjilkuri) in the flag leaf tissue (Fig. 4). For example, in Tamaroi, *ARF8* was consistently upregulated by water stress from booting to flowering (3 to 18 DAT) with a peak of *ARF8* upregulation at 12 DAT. A similar regulatory pattern was observed in the flag leaf of Yawa, except for a slight downregulation of *ARF8* at 15 DAT. In contrast, in the flag leaf of Tjilkuri, *ARF8* was consistently downregulated under stress from 3 DAT to 18 DAT. In EGA Bellaroi, *ARF8* was downregulated at 6, 9, 12 and 18 DAT, where the most apparent reduction was found at 15 and 18 DAT. In the head tissue, the regulatory pattern of *ARF8* fluctuated in all the durum varieties studied and could not be associated with the tolerant or sensitive nature of the genotype. At the start of water-deficit stress (3 DAT), *ARF8* was upregulated in the WG of Tamaroi and EGA Bellaroi, but was downregulated in Yawa and Tjilkuri. After 18 days of stress, *ARF8* was downregulated in the WG treatment of Tamaroi, Yawa and EGA Bellaroi but upregulated in Tjilkuri.

An inverted regulatory pattern between stress tolerant and sensitive varieties could also be found for *ARF18* expression in the flag leaf tissue of water stressed plants (Fig. 5). In Tamaroi and Yawa, *ARF18* was consistently upregulated from 3 DAT to 18 DAT, especially at 9 to 18 DAT for Tamaroi and 3 to 9 DAT for Yawa. In contrast, in the flag leaf of EGA Bellaroi and Tjilkuri, *ARF18* was consistently downregulated from 3 DAT to 18 DAT (except for EGA Bellaroi at 9 DAT when the fold change was marginal). The most apparent reduction of *ARF18* under stress in Tjilkuri was observed 3 DAT, while for EGA Bellaroi it was found at 12 DAT. In the developing head tissue, the regulatory pattern of *ARF18* is

subject to genotype. In Tamaroi, *ARF18* was consistently upregulated in the stress treatment from booting to flowering (except for a minimal fold change at 18 DAT). In Tjilkuri, *ARF18* was downregulated under stress from 3 DAT to 18 DAT except for 12 DAT. For Yawa and EGA Bellaroi, although the regulation of *ARF18* under stress fluctuated without a clear pattern, expression was generally upregulated in Yawa and downregulated in EGA Bellaroi.

An inverted regulatory pattern of miR160 between stress tolerant and sensitive varieties was also observed in the flag leaf (Fig. 6). For Tamaroi and Yawa, miR160 was downregulated under stress from 3 DAT to 18 DAT (except for Yawa at 9 DAT when a slight increase was detected). The most apparent downregulation of miR160 in Tamaroi under stress was found at 3 DAT, while in Yawa it was later (18 DAT). For EGA Bellaroi and Tjilkuri, in general, miR160 was upregulated from 3 DAT to 18 DAT (with exceptions at 3 DAT for EGA Bellaroi and at 6 DAT for Tjilkuri). In the developing head, the expression profile of miR160 is different for each genotype. For example, no obvious regulation of miR160 under stress was found in EGA Bellaroi from 3 DAT to 12 DAT, after which it was downregulated. In Tjilkuri, the response of miR160 to stress fluctuated across different time-points. Overall, in the flag leaf tissue, a negative correlation was found between miR160 (downregulation in the stress tolerant varieties, upregulation in the stress sensitive varieties) and its targets *ARF8* and *ARF18* (upregulation in the stress tolerant varieties, downregulation in the stress sensitive varieties). However, in the head tissue, such correlation was less clear and could only be found in certain genotypes at certain stress time-points (e.g. in Yawa at 12 DAT between miR160 and *ARF18*).

Discussion

Water-deficit stress is considered one of the main environmental factors limiting plant growth and crop yield worldwide, especially in rain-fed areas. Within the same crop species, genotypes can significantly differ in physiological and molecular stress response pathways (Rampino *et al.* 2006; Praba *et al.* 2009), consequently leading to differential yield performance under water-limiting conditions. The study of such genotypic differences contributes to our understanding of possible stress response mechanisms underlying stress tolerance, thereby providing traits or breeding targets for crop improvement under challenging environments. In this study, we focused on the genotypic water-deficit stress responses in stress tolerant and sensitive durum varieties, by examining physiological traits

and the miR160-*ARF*s regulatory module at different time-points of water-deficit stress, as well as harvest components and morphological traits at maturity. The three physiological parameters measured in this study were chlorophyll content, leaf relative water content, and stomatal conductance. Chlorophyll content reliably assesses photosynthetic activity as the photosynthetic potential of a plant directly depends on the quantity of chlorophyll present in the leaf tissue (Richardson *et al.* 2002) and therefore is a good indicator of water stress tolerance in terms of evaluating damage to the photosynthetic apparatus (Li *et al.* 2006; Anjum *et al.* 2011). Moreover, the measurement of chlorophyll content using a SPAD meter has the advantage of being non-destructive and rapid. Leaf relative water content directly reflects the cellular water status and osmotic potential in plants. Although destructive, using the penultimate leaf avoids damage to the flag leaf (Ma *et al.* 2006; Farooq *et al.* 2008) and is consistent with RWC in the penultimate leaf and the flag leaf of the same plant being similar (Ma *et al.* 2006). The stomatal conductance could differ between two sides (abaxial and adaxial) of the leaf tissue in cereals (Driscoll *et al.* 2006; Khazaei *et al.* 2010), with differential sensitivity to abiotic stress (James *et al.* 2008). Thus the stomatal response was evaluated on both leaf surfaces in this study. The miR160, *ARF8* and *ARF18* regulatory module, previously identified by our laboratory, was selected for its potential role in stress signalling and plant development (Liu *et al.* 2016b). Measurement of physiological traits and molecular regulatory modules at different time-points of stress treatment between booting and flowering were important to analyse, as this enabled how the early and late stress conditions are perceived by different durum varieties and their responses at different developmental stages to be measured. As the water-deficit stress continued to maturity, harvest components and morphological traits were evaluated to validate the stress tolerance level of these four varieties with regards to their agronomic performance. Significant reductions in grain number, fertile tiller number and total grain weight were only found under water stress in the two stress sensitive genotypes leading to yield loss, which is in accordance with previous findings where stress at the reproductive stage mainly inhibits fertility (Ji *et al.* 2010; Liu *et al.* 2015a).

Well-balanced physiological stress responses before anthesis could potentially contribute to the maintenance of grain number

In the present study, across the physiological parameters measured at different time-points, distinct genotypic responses to water-deficit stress are found between the stress tolerant and sensitive durum wheat varieties. In EGA Bellaroi and Tjilkuri (stress sensitive), water-deficit stress from booting to flowering caused reductions in the chlorophyll content, leaf relative water content and stomatal conductance. However, in the stress tolerant varieties Tamaroi and Yawa, only a minor decrease in leaf relative water content and chlorophyll content could be observed at the later stages of stress. The stomatal conductance of Tamaroi and Yawa exhibited a substantial drop at the start of the stress (3 DAT), similar to EGA Bellaroi and Tjilkuri but to a lesser extent. These results suggest that water-deficit stress possibly has immediate impacts on the transpiration activity due to stomatal movement, while chlorophyll content and leaf water status are gradually affected as the stress continues. The rapid response of stomatal closure could have been due to a stress-induced reduction in plant water status leading to the accumulation of ABA (abscisic acid), reduced cellular turgor and possibly inhibited osmotic adjustment in the guard cell (Brown *et al.* 1976; Schroeder *et al.* 2001; Luan 2002). Indeed, in EGA Bellaroi and Tjilkuri, leaf relative water content exhibited a similar immediate drop at the start of the stress (3 DAT). However, the changes of ABA level and osmotic potential in the guard cell and their association with relative water content and stomatal conductance under water-deficit stress require further investigation in durum wheat.

Another interesting genotypic pattern is that stronger positive correlations among the three physiological traits were found in the stress sensitive varieties. This suggests that the reductions of these physiological parameters in EGA Bellaroi and Tjilkuri synchronistically and negatively impacted plant fitness and development under water-deficit stress. Stress-induced reduction in the chlorophyll content indicates damage in the photosynthetic apparatus, possibly a direct consequence of oxidative damage by the stress-induced ROS (reactive oxygen species) in the leaves (Loggini *et al.* 1999; Munné-Bosch *et al.* 2001). In the control groups, as expected, the chlorophyll content generally increased from booting to flowering possibly to cater for the increased assimilate accumulation and photosynthetic requirement for reproduction (Corbesier *et al.* 1998; Inoue *et al.* 2004). However, in the stress sensitive varieties EGA Bellaroi and Tjilkuri, significantly reduced chlorophyll content under stress indicates possible damage to the photosynthetic apparatus (thus inhibiting photosynthetic activity), which is ultimately reflected in their inferior reproductive

performance (significantly reduced fertile tiller number and grain number). Furthermore, photosynthetic activity also relies on the carbon dioxide supply through the stomata. At later time-points of the stress (when flowering was starting), with the relative water content reaching 13-20% reduction in the stress sensitive varieties, the stomatal conductance was significantly impaired with almost complete closure on both of the leaf surfaces, especially for Tjilkuri. In the stress sensitive varieties, lowered availability of carbon dioxide as the result of stomatal closure, and the damage of photosynthetic apparatus due to low cell turgor, would both therefore inhibit photosynthetic capacity (Wong *et al.* 1979; Monneveux *et al.* 2006; Subrahmanyam *et al.* 2006; Yang *et al.* 2006b). Such photosynthetic inhibition during early reproductive development has been shown to affect pre-anthesis carbohydrate accumulation, causing irreversible negative impacts on reproductive organs, especially anthers (Inoue *et al.* 2004; Ji *et al.* 2010); thus explaining the significant reductions in the grain number and fertile tiller number observed in EGA Bellaroi and Tjilkuri.

Importantly, in Tamaroi and Yawa, tolerance may be a result of the maintenance of the photosynthetic apparatus and the coordinated control of the stomatal aperture. The rapid decline of stomatal conductance at 3 DAT could reduce water loss by transpiration, while unchanged chlorophyll content indicates the maintenance of photosynthetic capacity despite a reduced carbon supply. These results suggest that in the tolerant varieties, the stomatal movement was coordinated to the extent that photosynthesis remained unaffected while reducing water loss through the appropriate extent of stomatal closure. Moreover, there was no further reduction in stomatal conductance of the WG in Tamaroi and Yawa after 3 DAT. In fact, the adaxial stomatal conductance of the WG in Tamaroi was significantly higher at 18 DAT than at 3 DAT ($P < 0.05$). The maintenance of stomatal conductance could contribute to the carbon fixation ability and thus photosynthetic capacity (Wong *et al.* 1979; Monneveux *et al.* 2006; Subrahmanyam *et al.* 2006; Yang *et al.* 2006b), which is not only beneficial to carbohydrate storage at pre-anthesis but also reduces the need of pre-anthesis assimilate remobilisation as the stress progressed to post-anthesis, as shown previously in stress tolerant bread wheat (*Triticum aestivum*) (Inoue *et al.* 2004). Moreover, a maintained chlorophyll content is also associated with increased protective capacity against oxidative damage in the leaves, contributing to stress tolerance as studied in bread wheat (Chakraborty and Pradhan 2012; Gregorová *et al.* 2015). Therefore in the stress tolerant durum varieties, stomatal conductance balancing transpiration activity and the reservation of water contributed to the higher leaf relative water content and minimal damage to the photosynthesis apparatus.

Ultimately, the coordinated dynamics among these physiological parameters at different stages of pre-anthesis water-deficit stress would contribute minimal damage to the reproductive organs and spike fertility, leading to the maintenance of grain number and fertile tiller number at harvest in tolerant varieties.

Genotypic response of miRNA-mediated regulation could potentially contribute to coordinated stress signalling and adaptive physiological performance

Under environmental stress, plant developmental processes are adaptively modulated via the coordinated reallocation of metabolic resources across different physiological pathways, in order to maximise plant survival and fitness (Bohnert *et al.* 1995; Morsy *et al.* 2007; Tognetti *et al.* 2012). A range of stress signalling pathways mediated by growth hormones are involved in this process, including auxin signalling pathways. Auxin (indole-3-acetic acid, IAA) plays indispensable roles in almost all aspects of plant developmental processes, and mediates the hormone crosstalk in stress response mechanisms (Teale *et al.* 2006; Depuydt and Hardtke 2011). Under stress conditions, the abundance of auxin and auxin responsive genes at the cellular and molecular level mainly contribute to the plant stress acclimatisation via regulating the developmental plasticity, such as adaptive changes in organ pattern formation and tropism (Potters *et al.* 2007; Tognetti *et al.* 2012). Moreover, auxin has the advantage over other phytohormones for its ability to transport in long (source to sink) and short (cell to cell) distances (Friml 2003). Plant growth and development under abiotic stress largely depends on the spatiotemporal distribution of auxin and cellular auxin homeostasis (Tognetti *et al.* 2012). Furthermore, auxin receptors and auxin responsive genes could integrate various abiotic stress signals to modulate cellular responses to the variant auxin levels in different tissues which in turn provides feedback to affect auxin metabolism and transport (Ljung 2013).

On the molecular level, auxin signalling and metabolism are tightly regulated by many conserved plant miRNAs. The most important components in auxin signalling, TIR1/AFB family (auxin receptors), Aux/IAA proteins (transcriptional repressors), and ARF transcription factors (regulators of auxin responsive genes) are all directly or indirectly regulated by miRNAs (Sunkar *et al.* 2012; Liu *et al.* 2016a). Specifically, in the model species arabidopsis and several other crops, *ARF10, ARF16*, and *ARF17* are all targeted by miR160 family members, and such regulation appears to be important to adaptive shoot and

462 root development under abiotic stresses (Ding *et al.* 2009; Gutierrez *et al.* 2009; Guerra *et al.*
463 2015; Ma *et al.* 2015). The miR167 family targets *ARF6* and *ARF8*, to regulate reproductive
464 processes such as anther sterility and ovule development (Nagpal *et al.* 2005; Wu *et al.*
465 2006). In our previous study, RLM-RACE validated that durum miR160 targets both *ARF18*
466 and *ARF8* (Liu et al. 2016b). In arabidopsis, ARF18 is involved in female gametophyte and
467 ovule development (Pagnussat *et al.* 2009; Skinner and Gasser 2009; Shi and Yang 2011)
468 while in rapeseed it is associated with seed weight and silique length (Liu *et al.* 2015c).
469 Additionally, all these miRNA-*ARF*s regulatory modules have complex stress responsive
470 expression patterns under stress conditions (Jain and Khurana 2009; Tang *et al.* 2012; Liu *et*
471 *al.* 2016a). Interestingly, the pairing of miR160-*ARF8/18* also appears to be unique in durum
472 wheat (Liu *et al.* 2016b) (when compared to the pairing of miR167-*ARF8* in other plant
473 species). To further examine the interactions between miR160 and *ARF8/18* under stress,
474 their expression profiles were characterised in the present study among stress tolerant and
475 sensitive varieties at different time-points from booting to flowering.

476 Within each durum wheat genotype, complex temporal patterns of expression were
477 observed for both miR160 and *ARF*s across different time-points of stress from booting to
478 flowering. For example, in the head of Yawa, miR160 was downregulated (or unchanged)
479 under stress from 3 DAT to 12 DAT and at 18 DAT, but was upregulated at 15 DAT. In the
480 head of EGA Bellaroi, *ARF18* did not change under stress from 3 to 9 DAT in general, but
481 was downregulated from 12 to 18 DAT. In addition, there was no clear negative correlation
482 between the regulatory pattern of miR160 and *ARF8/18* under stress in the head tissue. Other
483 studies which reported on the fold-changes of miRNA regulatory modules at different stages
484 of stress treatment also identified such phenomena in expression patterns. For example, under
485 cold stress in bread wheat during spike development, tae-miR167c was downregulated at the
486 1.5 mm and 2 mm anther stages, but was substantially upregulated at the 3.0 mm anther stage
487 (meiotic division); while significant downregulation of miR167d was only found at the 1.5
488 mm anther stage (Tang *et al.* 2012). *ARF6* and *8*, targeted by tae-miR167 family members
489 also had a fluctuating regulatory pattern across different stages under cold stress (Tang *et al.*
490 2012), but without a clear negative correlation with their miRNA. Such temporal regulatory
491 patterns observed in the durum head tissue across different time-points of stress indicate that
492 miRNA and ARFs could not only play a role in stress responses, but also in plant
493 developmental processes such as anther development and fertilisation (Nagpal *et al.* 2005;
494 Goetz *et al.* 2007). Moreover, other regulatory mechanisms of ARFs might also be in effect

apart from miRNAs, such as the ubiquitin-mediated degradation of Aux/IAA proteins that allows for the function of ARF proteins (Gray *et al.* 2001), adding complexity to the auxin-regulated processes. However, such mechanisms require further investigation in durum wheat under water-deficit stress.

Most importantly, in the flag leaf tissue, the expression of miR160, *ARF8* and *ARF18* exhibited inverted regulatory patterns between stress tolerant and stress sensitive varieties, and negative correlations could be found between the miRNA-*ARF* pair. Overall, miR160 was downregulated in the two stress tolerant varieties but upregulated in the stress sensitive varieties while generally both of the *ARF*s were upregulated in the stress tolerant varieties but downregulated in the stress sensitive varieties (despite a few variations). As ARFs are crucial regulators within the auxin signalling pathways involved in many important aspects of plant development and stress adaptation, such genotypic miR160-*ARF* regulatory patterns might be contributing to stress tolerance on the physiological level. Specifically, ARF8 transcriptionally activates the auxin responsive *GH3* gene family (Yang *et al.* 2006a). *GH3* genes encode enzymes that adenylate IAA to form amino acid conjugates, therefore preventing the excessive accumulation of free auxin and achieving cellular auxin homeostasis (Staswick *et al.* 2005; Ludwig-Müller 2011). Plant total auxin exists in both free and conjugated forms, and the conjugation mechanism is a critical regulatory pathway to balance free active IAA and stored auxin conjugates (Korasick *et al.* 2013). Excessive accumulation of free IAA could result in phenotypic abnormalities and reproductive sterility (Bartel 1997), and the suppression of free IAA via promoting auxin conjugation could contribute to biotic and abiotic stress tolerance (Park *et al.* 2007; Ding *et al.* 2008; Domingo *et al.* 2009). In addition, GH3 appears to contribute to stress defence through its role in other plant hormone pathways such as salicylic acid and jasmonic acid signalling, via regulating hormone abundance by the adenylating reaction (Bari and Jones 2009; Jain and Khurana 2009). ARF18 is a positive regulator of auxin signalling by repressing IAA16 (INDOLE ACETIC ACID-INDUCED PROTEIN 16) (Oh *et al.* 2009). IAA16 belongs to the Aux/IAA family of transcriptional repressors, and the repression of Aux/IAA proteins is essential for normal auxin signalling (Worley *et al.* 2000; Rinaldi *et al.* 2012). A gain-of-function mutation in IAA16 substantially affected auxin responses and inhibited plant growth and sterility (Rinaldi *et al.* 2012). In the stress tolerant durum varieties, at different stages of water-deficit stress the increased level of *ARF8* and *ARF18* would lead to a higher level of GH3 and a decreased level of IAA16, thereby balancing auxin metabolism and enhancing auxin signalling under

stress. Adjusted auxin signalling in the leaf tissue under water deficit could also possibly contribute to source-to-sink auxin transport, thus modulating the reallocation of metabolic resources in the developing head (Cole and Patrick 1998; Yang *et al.* 2001; Xie *et al.* 2003). In rice (*Oryza sativa* L.) plants undergoing water-deficit stress during grain filling, altered hormonal balance in the head led to the remobilisation of carbon to the grains and a faster grain filling rate (Yang *et al.* 2001). In bread wheat, the ability to maintain IAA content under water-deficit stress contributed to photoassimilate translocation during grain filling and therefore less yield loss (Cole and Patrick 1998; Xie *et al.* 2003). However, in durum wheat, the relationship between miRNA-mediated auxin signalling in the flag leaf and its association with auxin levels in the reproductive tissues requires further investigation. In the leaf tissue, auxin homeostasis could also impact photosynthetic components and chloroplast metabolism (Volfová *et al.* 1978; Tognetti *et al.* 2010; Tognetti *et al.* 2012), thus contributing to physiological stress adaptation. In several plant species, different levels of auxin could either induce or reduce chlorophyll content and change chloroplast structure (Volfová *et al.* 1978; Fregeau and Wightman 1983; Tognetti *et al.* 2012). In arabidopsis under water stress, adaptive photosynthetic responses associated with energetic advantage and stress tolerance due to the ectopic expression of a UDP-glucosyltransferase (favouring auxin indole-3-butyric acid as substrate) in the transgenic plants could be simulated in wild-type plants by the supply of exogenous auxin (Tognetti *et al.* 2010). All these studies suggest that the photosynthetic responses contributing to stress tolerance in durum wheat could be associated with auxin homeostasis and coordinated auxin signalling mediated by miRNA-*ARF*s on the molecular level.

Another possible link between the miRNA-*ARF*s regulatory module and physiological adaptation centres on the role of auxin in hormone crosstalk. Auxin and cytokinin are known to antagonise the effects of abscisic acid (ABA) on stomatal closure (Tanaka *et al.* 2006). Under water-deficit stress, ABA plays an important role in the regulation of stomatal movement through affecting the guard cell osmotic potential (Wilkinson and Davies 2002). Thus an appropriate ratio of auxin and cytokinin could regulate the stomatal closure under water-deficit stress, coordinating the balance between reserving water via reducing transpiration and maintaining carbon supply for photosynthesis. A balanced ratio of auxin and cytokinin could also promote the formation of lateral roots, possibly contributing to enhanced water-uptake under stress (Lavenus *et al.* 2013). However, such links require further experimental validation in stress tolerant durum wheat varieties.

Conclusions

In summary, the present study shows the genotypic responses of different durum wheat varieties during different stages of water stress at the physiological and molecular level, which were ultimately reflected in their yield components. At the physiological level, stress tolerant durum varieties exhibit adaptive changes in traits like stomatal conductance and photosynthetic capacity to withstand stress more effectively than stress sensitive varieties. For all durum varieties studied, pre-anthesis water-deficit stress has an immediate impact on stomatal conductance but affects chlorophyll content and leaf water status gradually. At the molecular level, miR160 and its targets *ARF8* and *ARF18* exhibited dynamic and complex stress responsive patterns from booting to flowering, subject to the genotype. We propose that the distinct regulatory pattern of the miR160-*ARF*s module in two stress tolerant varieties contributes to coordinated auxin signalling and auxin homeostasis, possibly in association with their adaptive physiological traits. Together, water-deficit stress responses characterised in this study may have the potential to be used for stress tolerance screening and crop improvement in durum breeding programs.

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Figures

Fig. 1. Chlorophyll content (SPAD units) of four durum wheat genotypes at different time-points of pre-anthesis water-deficit stress. CG, control group; WG, water-deficit stress group; Ta, Tamaroi; Ya, Yawa; Be, EGA Bellaroi; Tj, Tjilkuri. Means ± SE are shown for n = 4 at each time-point.

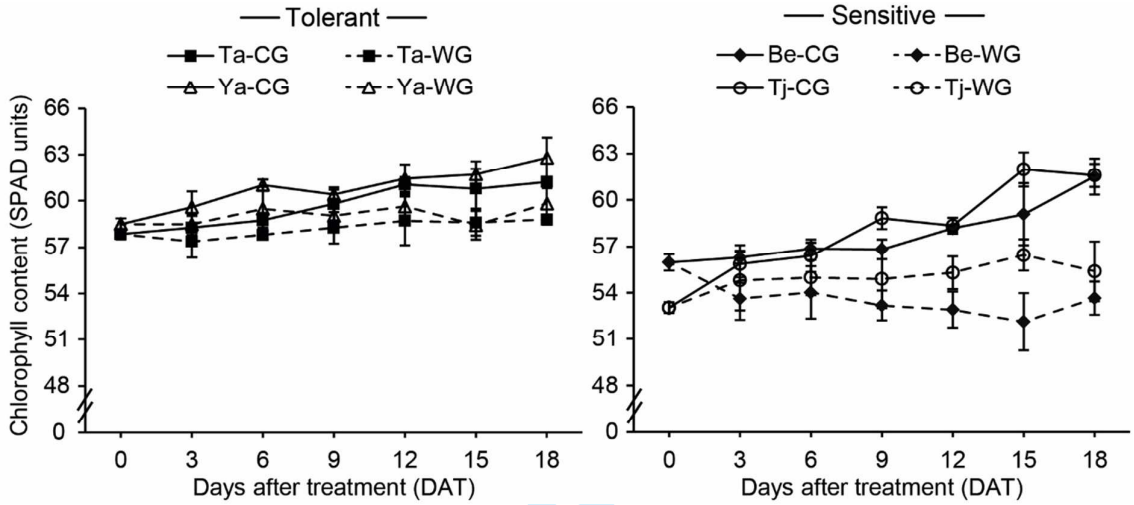
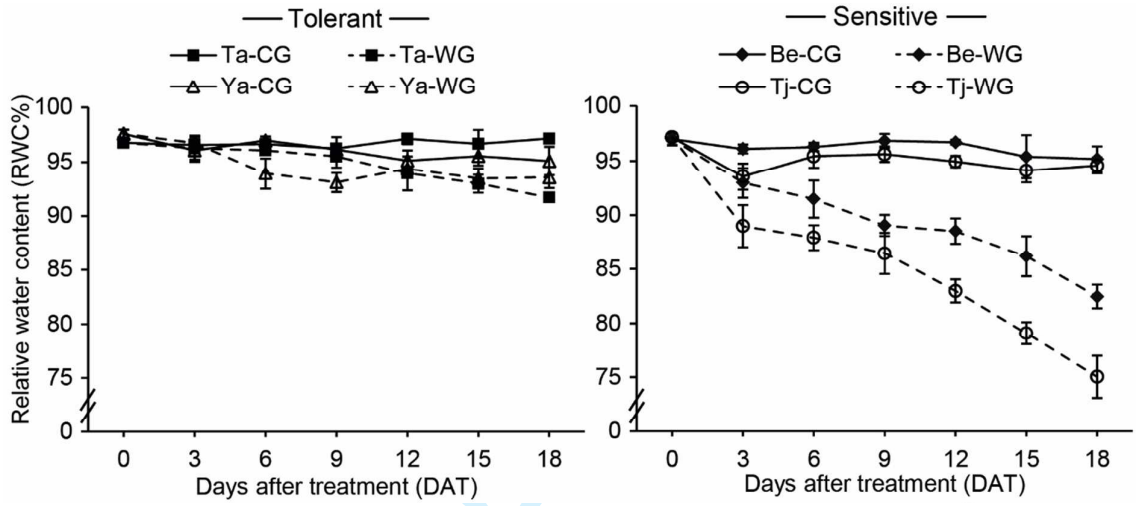


Fig. 2. Leaf relative water content (RWC%) of four durum wheat genotypes at different time-points of pre-anthesis water-deficit stress. CG, control group; WG, water-deficit stress group; Ta, Tamaroi; Ya, Yawa; Be, EGA Bellaroi; Tj, Tjilkuri. Means ± SE are shown for n = 3 at each time-point.



908 **Fig. 3.** Stomatal conductance (g_s, mmol m^{-2} s^{-1}) on the (*a*) adaxial leaf surface and (*b*) abaxial
909 leaf surface of four durum wheat genotypes at different time-points of pre-anthesis water-
910 deficit stress. CG, control group; WG, water-deficit stress group; Ta, Tamaroi; Ya, Yawa; Be,
911 EGA Bellaroi; Tj, Tjilkuri. Means ± SE are shown for n = 4 at each time-point.

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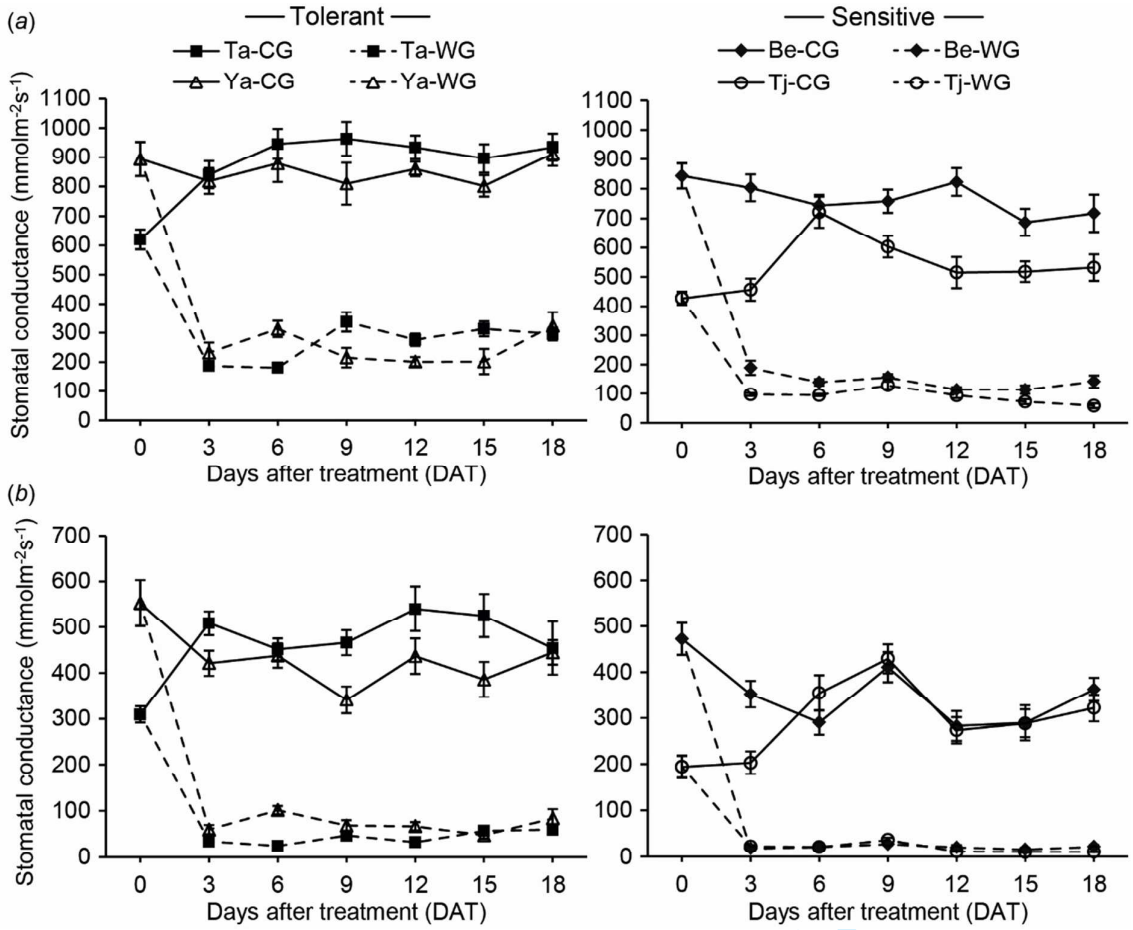

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915 **Fig. 4.** Differential expression of *ARF8* in response to pre-anthesis water-deficit stress in the
916 flag leaf and the developing head at different time-points in four durum wheat varieties.
917 DAT, days after treatment; FL, flag leaf; H, developing head; Ta, Tamaroi; Ya, Yawa; Be,
918 EGA Bellaroi; Tj, Tjilkuri. The bars represent the log (2)-fold changes (means ± SE for n =
919 3) between the CG (control group) and WG (water-deficit stress group).

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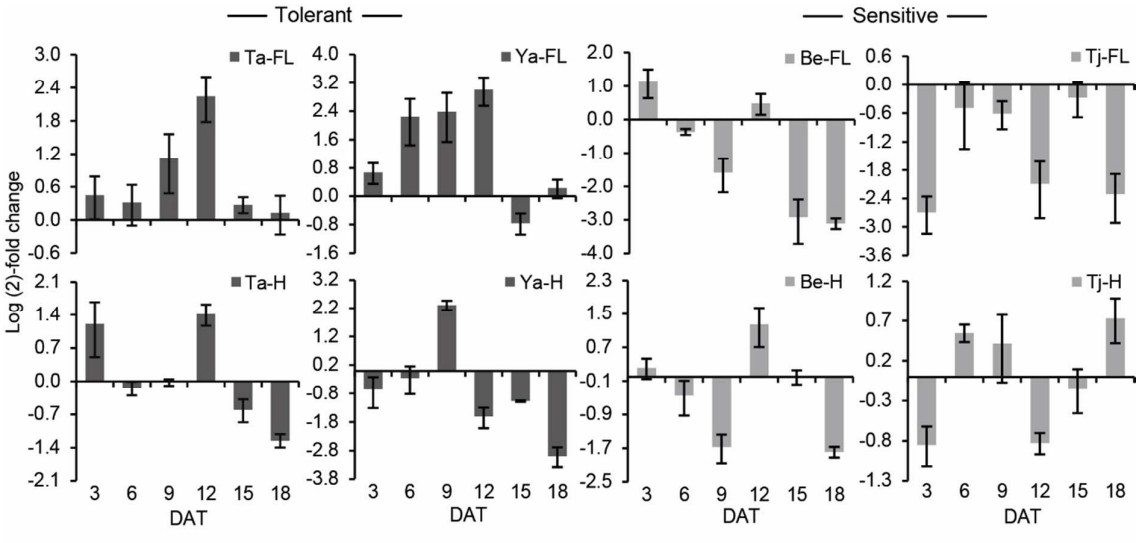

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Fig. 5. Differential expression of *ARF18* in response to pre-anthesis water-deficit stress in the flag leaf and the developing head at different time-points in four durum wheat varieties. DAT, days after treatment; FL, flag leaf; H, developing head; Ta, Tamaroi; Ya, Yawa; Be, EGA Bellaroi; Tj, Tjilkuri. The bars represent the log (2)-fold changes (means ± SE for n = 3) between the CG (control group) and WG (water-deficit stress group).

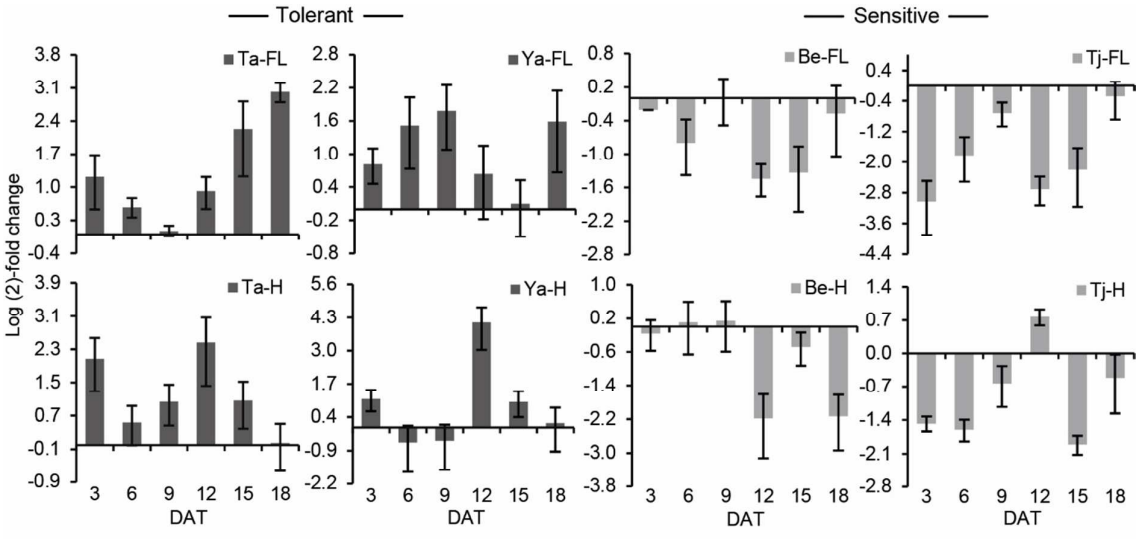
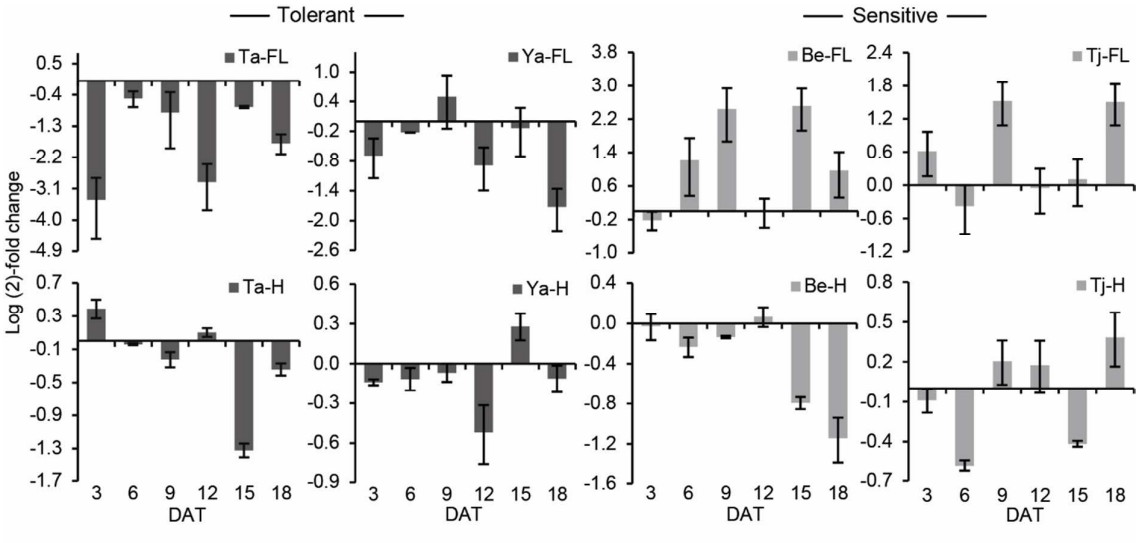


Fig. 6. Differential expression of miR160 in response to pre-anthesis water-deficit stress in the flag leaf and the developing head at different time-points in four durum wheat varieties. DAT, days after treatment; FL, flag leaf; H, developing head; Ta, Tamaroi; Ya, Yawa; Be, EGA Bellaroi; Tj, Tjilkuri. The bars represent the log (2)-fold changes (means ± SE for n = 3) between the CG (control group) and WG (water-deficit stress group).



936 **Tables**

937 **Table 1. Correlation coefficients (*r*) between chlorophyll content, leaf relative water**
938 **content and stomatal conductance (g_s) under pre-anthesis water deficit in stress tolerant**
939 **(*a*) and sensitive (*b*) durum wheat genotypes at 15 DAT (days after treatment)**

940

(*a*) Tolerant varieties	Relative water content	g_s - adaxial	g_s - abaxial
Chlorophyll content	0.51	0.56	0.54
Relative water content		0.68	0.61
g_s - adaxial			0.97
(*b*) Sensitive varieties	Relative water content	g_s - adaxial	g_s - abaxial
Chlorophyll content	0.50	0.66	0.73
Relative water content		0.87	0.77
g_s - adaxial			0.91

941 **Table 2. Effect of water-deficit stress on the morphological traits and yield components of four durum wheat genotypes** CG, control
942 group; WG, water-deficit stress group; Ta, Tamaroi; Ya, Yawa; Be, EGA Bellaroi; Tj, Tjilkuri. Means ± SE are shown for n = 4. * indicates the
943 statistical significance of $P < 0.05$ between the CG and WG for that variety

Variety	Plant height (cm)		Fertile tiller number		Main spike length (cm)		Biomass (g)		Grain weight (g)		Grain number		Harvest index	
	CG	WG	CG	WG	CG	WG	CG	WG	CG	WG	CG	WG	CG	WG
Ya	56.5±1.2	53.6±0.9	5.5±0.3	5.3±0.3	6.7±0.1	6.9±0.2	12.9±0.6	10.9±0.2*	5.2±0.2	4.6±0.2	155.0±7.8	134.8±7.3	0.41±0.02	0.42±0.01
Ta	57.4±1.1	54.4±0.7	4.5±0.3	4.3±0.3	7.3±0.3	7.7±0.1	13.1±1.0	11.9±0.4*	5.2±0.5	4.7±0.2	136.8±11.5	124.8±5.5	0.40±0.01	0.40±0.00
Tj	52.2±1.0	48.2±0.8*	4.3±0.3	2.8±0.3*	6.6±0.2	6.5±0.1	13.8±0.3	7.2±0.4*	4.8±0.4	1.2±0.2*	132.3±6.3	39.8±6.8*	0.35±0.02	0.16±0.01*
Be	54.8±0.9	53.4±0.8*	4.0±0.4	2.3±0.3*	6.8±0.1	6.7±0.2	11.9±0.5	8.9±0.5*	4.6±0.3	1.7±0.4*	112.5±7.1	40.3±10.2*	0.38±0.01	0.19±0.04

944

945

946 **Supplementary material**

947 **Table S1. qPCR primers used in this study**

948

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
Auxin response factor 8	CATTATCATCACACCGACAGCTAC	GGGTAAGGTGGAGATCCGATAAA
Auxin response factor 18	CCTATGCTGTTACTCGGACAA	TGAGCACAAAGCCCTTAGGTA
GAPDH	CTTCCAGGGTGACAACAGGT	GTGCTGTATCCCCACTCGTT
miR160	CTGGCTCCCTGTATGCCAAA	Universal qPCR primer [a]

949 [a] Provided in the MystiCq microRNA cDNA Synthesis Mix Kit (Sigma-Aldrich, Australia).

950 **Table S2. Correlation coefficients (*r*) between yield components and morphological traits in four durum wheat genotypes**

951

	Fertile tiller number	Main spike length	Biomass	Grain weight	Grain number	Harvest index
Plant height	0.61	0.53	0.73	0.74	0.68	0.67
Fertile tiller number		0.28	0.69	0.82	0.89	0.83
Main spike length			0.41	0.47	0.40	0.46
Biomass				0.93	0.89	0.78
Grain weight					0.97	0.95
Grain number						0.93

952

Chapter 7

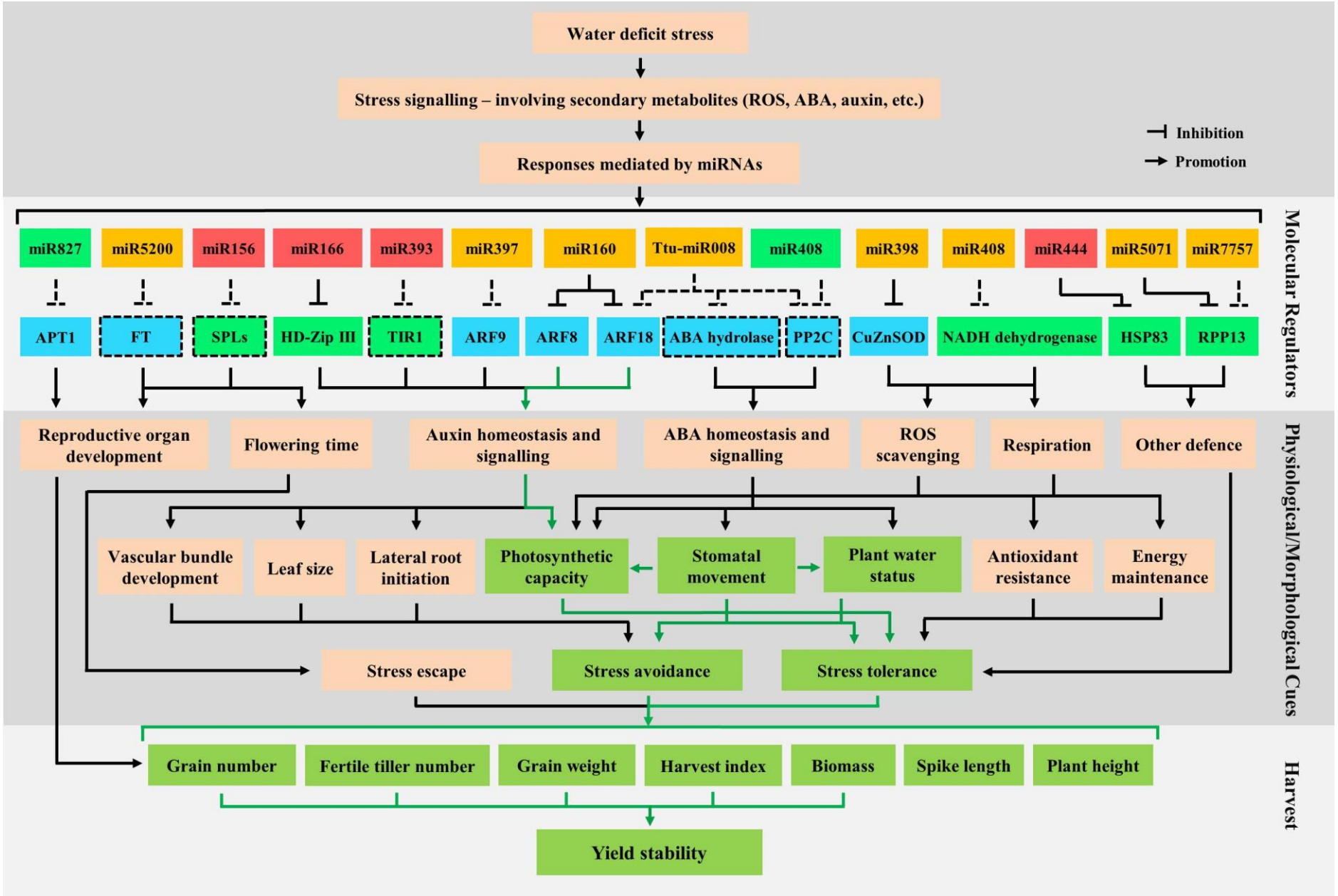
General Discussion

Chapter 7 General Discussion

Durum wheat (*Triticum turgidum* L. ssp. *durum*, AABB, 2n = 4x = 28) is considered to be the most agro-economically important tetraploid cereal species, especially in the Mediterranean region (Pecetti & Annicchiarico 1998; Annicchiarico *et al.* 2005). Given the damaging effects of water-deficit stress on the global production of durum wheat, a comprehensive understanding of the stress response mechanisms contributing to water-deficit tolerance is essential to support breeding strategies aiming for yield improvement in rain-fed areas (Fischer & Maurer 1978; Mohammadi *et al.* 2010; Nouri *et al.* 2011). In cereals, microRNAs (miRNAs) are central to post-transcriptional and translational regulation of gene expression in a variety of biological processes such as reproductive development and the responses to multiple abiotic factors including water deficit (Budak *et al.* 2015a; Liu *et al.* 2016a). More importantly, within the same crop species, comparative analysis of miRNA-mediated responses between stress tolerant and sensitive varieties have revealed unambiguous differential regulatory patterns, suggesting possible contributions to the stress tolerance level (Barrera-Figueroa *et al.* 2011; Wang *et al.* 2013; Ma *et al.* 2015). However, knowledge of cereal miRNA-associated stress response mechanisms has been mostly limited to rice and bread wheat, and such understanding in durum wheat was elusive. Thus, the primary objective of this project was to identify durum miRNAs and their functional target genes involved in pre-anthesis water-deficit stress responses in different durum varieties, and their association with key physiological parameters, morphological traits and yield components. This was achieved through four main modules (Chapters 3 through to 6) involving components such as glasshouse experiments investigating the physiological, morphological and yield components of different durum genotypes under water-deficit stress (20 genotypes in Chapter 3 and four genotypes in Chapter 6); high-throughput sequencing of the durum miRNA transcriptome across 96 small RNA libraries (Chapter 4); genome-wide *in silico* analysis of the target transcriptome of novel and conserved

durum miRNAs (Chapter 4 and 5); and experimental examination (5′ RLM-RACE and qPCR) of multiple miRNA-target pairs in stress tolerant and sensitive durum varieties (Chapters 4 through to 6), with specific temporal co-expression analysis of the miR160-ARFs (Auxin Response Factors) module and its association with physiological traits at different time-points of stress between booting and flowering (Chapter 6). From the combined findings, a model of water-deficit stress response mechanisms mediated by key durum miRNAs, in association with physiological and morphological traits and ultimately yield components can be proposed (Figure 7.1), as a preliminary template towards further research. Future opportunities and potential strategies of utilising the current findings in breeding programs, with a major focus on improving stress tolerance, are also outlined in the break-out boxes.

Figure 7.1 Proposed model of water-deficit stress response mechanisms mediated by key microRNA (miRNAs) in durum wheat. In the Molecular Regulators section: green boxes, upregulated miRNAs or targets under stress; red boxes, downregulated miRNAs or targets under stress; blue boxes, miRNAs or targets upregulated in the stress tolerant varieties but downregulated in the sensitive varieties; orange boxes, miRNAs or targets downregulated in the stress tolerant varieties but upregulated in the sensitive varieties. Dotted lines indicate *in silico* target analysis results, however further experimental work (5′ RLM-RACE for target degradation and qPCR for expression level) is required. Light green arrows and boxes in the Physiological/Morphological Cues and the Harvest sections represent components measured in this study. ABA, abscisic acid; APT1, aberrant pollen transmission 1; ARF, auxin response factor; CuZnSOD, copper-zinc superoxide dismutase; FT, flowering locus T; HD-Zip III, class III homeodomain-leucine zipper protein; HSP83, heat shock protein 83; PP2C, protein phosphatase 2C; ROS, reactive oxygen species; RPP13, disease resistance proteins RPP13; SPL, squamosa promoter-binding-like; TIR1, Transport Inhibitor Response 1.


Water deficit stress
Stress signalling – involving secondary metabolites (ROS, ABA, auxin, etc.)
Responses mediated by miRNAs
Inhibition
Promotion
Molecular Regulators
miR827
miR5200
miR156
miR166
miR393
miR397
miR160
Ttu-miR008
miR408
miR398
miR408
miR444
miR5071
miR7757
APT1
FT
SPLs
HD-Zip III
TIR1
ARF9
ARF8
ARF18
ABA hydrolase
PP2C
CuZnSOD
NADH dehydrogenase
HSP83
RPP13
Physiological/Morphological Cues
Reproductive organ development
Flowering time
Auxin homeostasis and signalling
ABA homeostasis and signalling
ROS scavenging
Respiration
Other defence
Vascular bundle development
Leaf size
Lateral root initiation
Photosynthetic capacity
Stomatal movement
Plant water status
Antioxidant resistance
Energy maintenance
Stress escape
Stress avoidance
Stress tolerance
Harvest
Grain number
Fertile tiller number
Grain weight
Harvest index
Biomass
Spike length
Plant height
Yield stability

During water-deficit stress, the water status of durum plants declines [indicated by leaf water potential and relative water content (RWC)] when soil moisture becomes limiting, possibly due to the reduced water availability for root water uptake (Barraclough *et al.* 1989). This could lead to reduced cell turgor triggering subsequent stress signals involving secondary stress metabolites such as ROS (reactive oxygen species) and various hormones [ABA (abscisic acid), auxin, ethylene, etc.] (Cruz de Carvalho 2008; Anjum *et al.* 2011; Peleg & Blumwald 2011). Excess accumulation of certain metabolites such as ROS has detrimental effects on plant growth and development, such as damage to the photosynthetic apparatus in the leaves (Loggini *et al.* 1999; Munné-Bosch *et al.* 2001; Miller *et al.* 2010). Other stress-associated metabolites such as ABA could induce stomatal closure via changes of the osmotic potential in the guard cell (Schroeder *et al.* 2001; Luan 2002; Wilkinson & Davies 2002). Even though a lowered stomatal aperture could reduce water loss from the transpiration activity, photosynthesis could also be inhibited due to a reduced carbon dioxide supply (Wong *et al.* 1979; Monneveux *et al.* 2006; Subrahmanyam *et al.* 2006; Yang *et al.* 2006). During early reproductive development, impairment of photosynthesis and pre-anthesis carbohydrate storage have irreversible detrimental effects on reproductive fertility (Inoue *et al.* 2004; Ji *et al.* 2010); thus ultimately causing spike sterility and grain number reduction responsible for yield loss (as observed for the stress sensitive durum varieties in Chapters 3 and 6).

Similar to other plant species, durum wheat has evolved sophisticated mechanisms to cope with water-deficit stress, and such mechanisms appear to be genotype-dependent. In plants, three main defence strategies against water-deficit stress (Levitt 1980) are stress escape, mainly involving developmental plasticity (e.g. early flowering and early maturity); stress avoidance, characterised by the maintenance of high tissue water status (e.g. decreased stomatal conductance to reduce water loss, increased lateral roots to enhance water uptake); and finally stress tolerance, represented by minimal cellular damage despite low cell turgor (e.g. enhanced

antioxidant resistance and reallocation of metabolic resources) (Levitt 1980; Richards *et al.* 2002; Simova-Stoilova *et al.* 2009). Some of these mechanisms are reflected in the alterations of physiological traits (e.g. chlorophyll content, stomatal conductance, leaf relative water content, etc.) under pre-anthesis water-deficit stress in durum wheat under glasshouse conditions, subject to genotype. Essentially, in the stress tolerant durum varieties, the maintenance of plant water status (stress avoidance strategy), minimal damage in the photosynthetic components (stress tolerance strategy), and effective control of stomatal conductance to balance water loss and carbon fixation for photosynthesis (stress avoidance and tolerance strategies); are the major attributes contributing to maintenance of reproductive fertility (fertile tiller number and grain number), and therefore yield stability.

Under abiotic stress, physiological and morphological cues are coordinated by the stress-regulated modification of gene expression on the molecular level (Chinnusamy *et al.* 2004; Cramer *et al.* 2011). miRNAs could rapidly respond to both stress and developmental cues, fine-tuning the gene expression of their cognate targets to coordinate the limited resources between different physiological pathways (Sunkar *et al.* 2012; Ding *et al.* 2013). In the four Australian durum varieties studied (two stress tolerant and two stress sensitive), pre-anthesis water-deficit stress caused complex and dynamic changes of miRNA expression (or even produced new miRNAs) in the flag leaf and the developing head. Via high-throughput sequencing and qPCR analysis (Chapters 4 and 5), a comprehensive description of the overall durum miRNA population (110 conserved and 159 novel) across different genotypes was provided, with stress-responsive, tissue-type and/or genotype dependent regulatory patterns revealed for most of the conserved miRNAs. In general, stress-reduced miRNAs could lead to the accumulation of positive regulators of stress adaptation, while stress-induced miRNAs could lead to the repression of the negative targets of stress adaptation (Khraiwesh *et al.* 2012;

Shriram *et al.* 2016). Future opportunities to exploit these miRNAs in durum wheat exist (Break-out Boxes 1 and 2).

Future Opportunity #1: Towards SMARTER durum breeding – phenotyping with miRNAs

Water-deficit stress tolerance is a key breeding objective in many cereal breeding programs around the world. The studies of stress-associated durum miRNAs have been limited to a small number of varieties so far. Expanding our knowledge in this key area through the evaluation of extensive germplasm collections will contribute to unravelling the practical value of miRNAs in breeding. Experimentation based on this objective, both in the laboratory, glasshouse and field conditions, could be conducted using several different approaches:

(a) Deep sequencing of the miRNA transcriptome in extensive panels of elite cultivars, breeding lines, landraces, and their wild progenitors could efficiently capture the global alteration of the miRNA population and their functional divergence during the domestication process.

(b) Large-scale co-expression analysis of key miRNA-target modules with qPCR in a wide range of germplasm focusing on genotype-environment interactions will enable systematic and accurate comparisons of the variation in their regulatory patterns.

(c) Hybridisation-based microarrays of miRNAs associated with stress tolerance could be a highly efficient screening method to detect the presence of such miRNAs in different tissues under stress across breeding lines.

Ultimately, this future opportunity would potentially enable breeding programs to reliably screen germplasm within their collections for miRNA-associated stress tolerance characteristics. Further development of miR-markers could also improve the efficiency of crossing strategies used by breeding programs, which is traditionally one of the most time-consuming components during the development of new varieties.

Future Opportunity #2: The potential of novel miRNAs – what do they have to offer?

Chapter 4 was the first report of novel miRNAs identified using a deep sequencing strategy in durum wheat. Further investigation of these novel miRNAs could provide valuable information on the miRNA regulatory pathways specific to durum. A systematic pipeline could be adopted here:

(a) Within the sequencing reads obtained across 96 small RNA libraries (Chapter 4), statistical analysis could be conducted to investigate the stress-responsive, tissue- and/or genotype-dependent expression patterns of these novel miRNAs.

(b) Genome-wide *in silico* investigation (with tools such as psRNATarget and Blast2GO) of the target repertoire of all novel durum miRNAs will reveal miRNA-mediated biological processes potentially unique in durum wheat.

(c) Experimental verification (qPCR, 5′ RLM-RACE, etc.) of predicted novel miRNA-target pairs in a wide range of durum wheat varieties will help characterise their interaction patterns subject to genotype during stress response(s).

Essentially, this future opportunity could enable the dissection of particular species-specific miRNA network components in durum wheat. Further comparison of novel miRNA-target modules across different cereal species could also identify miRNAs with evolutionarily distant roles during the speciation process.

Genome-wide *in silico* target analysis in Chapters 4 and 5 revealed a wide spectrum of functional genes (a total of 2186) targeted by conserved (113) and novel (4) durum miRNAs. Specifically for the stress responsive durum miRNAs, their target repertoire includes a broad range of proteins related to stress perception and plant development, such as various transcription factors, detoxifying enzymes and hormone signal transducers, placing durum miRNAs at the centre of the gene regulatory networks. Moreover, qPCR profiling and 5′ RLM-RACE examination of miRNA-target pairs under pre-anthesis water-deficit stress provided the first experimental evidence of miRNA-target interactions in durum wheat. Further experimental work could be extended as described in Break-out Box 3.

Future Opportunity #3: Pairing miRNAs and targets – more than meets the eye

The complete understanding of miRNA functions largely depends on the precise identification of their *bona fide* targets. However, to date there has been no other report of the experimental validation of miRNA-target pairing in durum apart from the collection of papers that have been published as a result of this project. Moreover, for certain durum miRNAs, no targets could be retrieved *in silico* possibly due to the limited genome information. Several approaches could be employed here to explore this future opportunity:

(a) The assembly of a custom *Triticum turgidum* L. ssp. *durum* transcriptome with up-to-date EST (Expressed Sequence Tag) information will significantly improve the accuracy and efficiency of *in silico* target analysis. Less stringent parameters should be applied to allow for non-canonical targets with low sequence homology, which is common to the miRNA-induced translational inhibition mechanism.

(b) A large scale 5′ RLM-RACE screening of miRNA-induced degradation extending to the targets predicted in other recent studies (such as Akpinar et al. 2015) would certainly capture more evidence of miRNA-target interactions in durum.

(c) A degradome sequencing approach (modified from 5′ RLM-RACE) could efficiently sequence millions of the uncapped ends of mRNA fragments in parallel. Combined with (a), this could be utilised to gain a global profile of miRNA-cleaved mRNAs and retrieve target information of previously unmatched miRNAs.

Future research focusing on the precise annotation and experimental validation of durum miRNA-target pairing on a genome-wide scale would extend the general view of durum miRNA networks and possibly reveal new genetic factors with implications in stress tolerance.

By targeting these genes with stress and/or development-associated implications, stress responsive durum miRNAs (individually or acting in accordance with other miRNA members with functional interplay) could regulate a wide range of biological processes (as shown in Figure 7.1), including:

1) auxin homeostasis and signalling [e.g. miR160-ARF8/18, miR397-ARF9, miR393-TIR1 (Transport Inhibitor Response 1) and miR166-HD-Zip III (class III homeodomain-leucine zipper protein)];

2) ABA metabolism and signalling [e.g. miR408-PP2C (protein phosphatase 2C) and Ttu-miR008-ABA 8′ hydrolase];

3) antioxidant defence and respiratory adjustment [e.g. miR398-CuZnSOD (Copper-zinc superoxide dismutase) and miR408-NADH dehydrogenase];

4) cellular metabolic processes (e.g. miR395-ATP sulfurylase and miR528-sucrose synthase);

5) reproductive events [e.g. miR5200-FT (flowering locus T), miR156-SPL (squamosa promoter-binding-like) and miR827-APT1 (aberrant pollen transmission 1)];

6) other defence mechanisms [e.g. miR444-HSP83 (heat shock protein 83) and miR5071-DRPs (disease resistance proteins)].

Under water-deficit stress, the reprogramming of the above biological processes modulated by durum miRNAs could potentially contribute to stress avoidance and/or tolerance strategies that enable the maintenance of yield components at harvest. For instance, in the stress tolerant durum varieties, stress-reduced expression of miR160 in the flag leaf from booting to flowering allowed for the accumulation of *ARF8* and *ARF18* (Chapter 6). Both ARF8 and ARF18 transcription factors are positive regulators of auxin homeostasis and auxin signalling via the promotion of auxin-responsive gene *GH3* (by ARF8) and repression of the auxin-responsive gene *IAA16 (indole-3-acetic acid 16,* by ARF18) (Worley *et al.* 2000; Staswick *et al.* 2005; Oh *et al.* 2009; Ludwig-Müller 2011; Rinaldi *et al.* 2012). The increased abundance of *ARF8*/*18* could contribute to a balanced ratio of conjugated/free auxin levels and enhanced auxin signalling, potentially leading to minimal damage of the photosynthetic components (stress tolerance), coordinated stomatal aperture (stress avoidance and tolerance), and possibly increased lateral roots to enhance water uptake (stress avoidance) (Tanaka *et al.* 2006; Bari & Jones 2009; Tognetti *et al.* 2010; Tognetti *et al.* 2012). However, in stress sensitive durum varieties, the regulatory patterns of miR160-ARF8/18 were inverted, suggesting that this stress defence pathway was not activated. Therefore, genotype-dependent regulatory patterns of particular miRNA-target modules between the stress tolerant and sensitive durum varieties

(Figure 7.1, blue and yellow boxes) represent a complex layer of genetic mechanisms determining the water-deficit stress tolerance level. Nonetheless, specific roles of certain durum miRNAs (especially novel miRNAs) and their *in silico* identified targets involving interrelated regulatory pathways require further experimental elucidation in different durum wheat genotypes (Break-out Box 4).

Future Opportunity #4: Deciphering the functional significance of miRNA machinery

Similar to other plants, a durum miRNA could target multiple genes and *vice versa*, adding complexity to the miRNA regulatory networks. However, direct functional dissection of miRNA-target modules in durum has been limited. Newly emerging RNA interference technologies could be utilised for this future opportunity.

(a) Genetic manipulation of miRNA abundance with gain-of-function (increasing miRNA expression) or loss-of-function (reducing or abolishing miRNA expression) methods, in conjunction with the evaluation of downstream biological changes is probably the most effective way to investigate the functional roles of a miRNA. Gain-of-function could be achieved via MIR gene overexpression or custom-made artificial miRNAs (amiRNAs) generated by replacing the miRNA duplex region in endogenous miRNA precursors with miRNAs of interest. Loss-of-function could be achieved via MIR gene knock-down, short tandem target mimics and target mimics designed to sequester miRNA activity, and point mutations in the miRNA/mRNA binding region to disrupt their interaction.

(b) When miRNA-target pairing is experimentally confirmed (through degradome sequencing or 5’ RLM-RACE), miRNA functions could also be determined by modifying an individual target gene alone. Target gain-of-function could be achieved via gene overexpression, while target loss-of-function could be achieved via amiRNAs which are designed based on any part of the target transcript.

Functionally beneficial miRNAs/targets raised from this future opportunity will enable the genetic engineering of desired agronomic traits in molecular breeding.

In conclusion, under pre-anthesis water-deficit stress, stress responsive durum miRNAs are central to the reprogramming of gene expression on the molecular level, contributing to the adaptive changes in the physiological and morphological cues, which are ultimately reflected in the yield components. Findings from this project provide new insight into durum miRNA-mediated water stress response networks, presenting more options to cereal research and

breeding programs with the ultimate goal of developing high-yielding elite varieties under adverse environments. Future research opportunities include phenotyping extensive durum germplasm with miRNAs (Break-Out Box 1), collective assessments of the novel durum miRNA machinery (Break-Out Box 2), global verification of miRNA-target pairing (Break-Out Box 3), functional deciphering of specific miRNA candidates for trait manipulation (Break-Out Box 4), and the examination of transgenerational stress tolerance conferred by miRNAs in breeding (Break-Out Box 5).

Future Opportunity #5: Transgenerational inheritance of miRNA stress tolerance

Plant miRNAs can provide stress memory to recurring abiotic stress within the generation (Stief *et al.* 2014). However, whether miRNA-conferred stress tolerance is inherited in the progenies of the water-stressed durum plants is unknown. To answer this question, the following strategies could be adopted:

(a) Comparative analysis of the miRNA transcriptome in the somatic (e.g. young leaves, flag leaf, and roots) and reproductive tissues (e.g. pollen, unfertilised ovules, embryo and endosperm) of stressed and non-stressed durum plants (in a panel of genotypes with varying stress tolerance), and in the same tissues of their stress-treated and untreated progenies. This could provide novel information about the miRNA-associated mechanisms underlying heritable tolerance, and how they are transmitted to the next generation during meiosis.

(b) The above comparative analysis could utilise various miRNA profiling methods for different purposes (e.g. high-throughput sequencing to gain the global miRNA population profile, qPCR to precisely quantify the abundance of certain miRNAs, and miRNA microarrays to quickly capture tissue-, stress-, genotype-specific miRNAs).

(c) The interaction between miRNAs and their targets should also be compared across generations in the materials mentioned in (a) to detect any differences in their regulatory patterns (using methods like qPCR, 5′ RLM-RACE, northern-blotting, etc.).

(d) All of the above should be investigated along with the screening of physiological traits that are reliable indicators of stress tolerance (e.g. leaf water potential, photosynthetic rate, etc.), a histological study of reproductive tissues (e.g. pollen, ovary, endosperm), and yield components to evaluate their associations.

Elucidating the contribution of miRNAs to transgenerational stress tolerance and their distribution among reproductive tissues may provide novel strategies towards trait-focused selection, convergent and divergent crossing, and heterotic-hybrid breeding.

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