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

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Abstract. In Mediterranean environments, water-deficit stress that occurs before anthesis significantly limits durum wheat (*Triticum turgidum* L. ssp. *durum*) production. Stress tolerant and stress 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) that 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 are 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).

Additional keywords: auxin response factors, *Triticum turgidum*.

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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 before 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*. 2015*a*). 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 with stress sensitive genotypes (Liu *et al*. 2015*a*). 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.

At 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 (reviewed by Liu *et al*. 2016*a*). 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*. 2016*a*). In our previous studies (Liu *et al*. 2015*b*, 2016*b*), 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*. 2016*b*). The phytohormone auxin regulates 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 at 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 thaliana* (L.) Heynh. and several other species, *ARF6* and *ARF8* are targeted by miR167 (Wu *et al*. 2006; Liu *et al*. 2014), whereas *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*. 2016*b*). miR160 has been reported to be water-deficit stress responsive in several cereal species including durum wheat (Liu *et al*. 2016*a*). 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 stress 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.

Materials and 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; whereas EGA Bellaroi and Tjilkuri are water-deficit stress sensitive (Liu *et al*. 2015*a*). 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*. 2015*a*) in a controlled environmental growth chamber at 22°C/16°C day/night temperature with a 12 h photoperiod, 45% RH. Briefly, all plants were well watered to field capacity (12% soil water content (SWC)) from germination to booting stage. At booting, water was withheld for the water-deficit stress group (WG) and SWC dropped to 6% within 24 h. From booting, SWC of the 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*. 2015*a*). 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 days after treatment (DAT)). For each sampling point, three flag leaf samples and three head samples were taken from three individual biological replicates (each from a different pot). 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) 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*. 2015*a*). Fresh leaves were sampled and weighed immediately to record FW. Leaves were then placed in distilled water for 5 h in the dark and weighed again to record turgid weight (TW). 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).

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*. 2015*a*) with four individual biological replicates (each from a different pot) 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) 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). 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) 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) on a ViiA7 Real-Time PCR machine (Applied Biosystems). 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.* 2016*b*). For the amplification of miR160, a forward miRNA-specific primer was designed based on the full mature miRNA sequence (as previously used to show specificity of miR160a to the targets *ARF8* and *ARF18*) (Liu *et al.* 2016*b*) 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. GAPDH was used as the reference gene for its stable expression across the durum wheat samples under water-deficit stress, which has been successfully used in previous studies for the quantification of durum miRNAs and their target genes including the ones studied in this report (Liu *et al.* 2015*b*, 2016*b*).

Statistical analysis

Statistical analysis of glasshouse data was performed as described previously (Liu *et al.* 2015*a*) using GENSTAT 15th edn (VSN International Ltd). Briefly, Student's *t*-tests ($P < 0.05$) were performed to detect the significant changes between CG and WG in physiological traits (chlorophyll content, RWC and stomatal conductance), morphological traits (plant height, fertile tiller number and main spike length) and yield components (grain weight, grain number, biomass and harvest index) in response to water-deficit stress for each genotype. One-way ANOVA was performed to determine differences between means for chlorophyll content, RWC and stomatal conductance across different time-points within each genotype using the least significance difference (l.s.d.) at $P < 0.05$. 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 ± s.e.) between the WG and CG at different time-points of stress were calculated for each genotype (Liu *et al.* 2016*b*). Student's *t*-tests ($P < 0.05$) were performed to detect the significant differences between the gene expression in CG and WG for each genotype. One-way ANOVA was performed to determine differences between mean gene expression fold-change across different time-points within each genotype using the least significant difference (l.s.d.) at $P < 0.05$.

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 exhibited an increasing trend from booting (0 DAT) to around anthesis stage (18 DAT) (Fig. 1) but the chlorophyll content at 18 DAT was only significantly higher than at 0 DAT for EGA Bellaroi ($P < 0.05$) and Tjilkuri ($P < 0.05$). At each time-point of water-deficit stress, the chlorophyll content of two stress tolerant genotypes (Tamaroi and Yawa) appeared to be lower in the WG compared with 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 of stress for EGA Bellaroi and at 9, 12, 15 and 18 DAT for Tjilkuri (Fig. 1).

For leaf RWC, its value in the CG of each genotype is similar (ranging from 94 to 98%) from booting to anthesis with no significant change observed ($P > 0.05$) (Fig. 2). For stress tolerant genotypes, the RWC appears to be lower in the WG when compared with the CG, but no significant difference was detected (except for 18 DAT in Tamaroi, $P < 0.05$). At 18 DAT, the average RWC in the WG of Tamaroi was 91.7% (compared with 97.2% in the CG), whereas the average RWC in the WG of Yawa was 93.6% (compared with 95.1% in the CG). However, a significant reduction of RWC ($P < 0.05$) between WG and CG plants was observed in both EGA Bellaroi and Tjilkuri at 6, 9, 12, 15 and18 DAT, with an even higher reduction in Tjilkuri. At 18 DAT, the RWC of the stressed EGA Bellaroi plants dropped to 82.5% (12.7% lower than the CG) ($P < 0.05$). For Tjilkuri, the RWC of the WG treatment was 75.0% (19.6% lower than CG) ($P < 0.05$).

Comparisons of stomatal conductance between the control and stress treatments in the four durum varieties were made on both adaxial (Fig. 3*a*) and abaxial surfaces (Fig. 3*b*). Overall, stomatal conductance on the abaxial surface of the flag leaf appeared to be more sensitive to stress than the adaxial surface, regardless of genotype. In addition, the two stress tolerant genotypes (Tamaroi and Yawa) showed less reduction in stomatal conductance on both abaxial and adaxial surfaces, compared with the two stress sensitive genotypes (EGA Bellaroi and Tjilkuri). Specifically, at 18 DAT, the adaxial stomatal conductance of the two stress tolerant genotypes, Tamaroi and Yawa, was 68.3% ($P < 0.05$) and 64.4% ($P < 0.05$) lower in the WG treatment than the controls respectively. However, the adaxial stomatal conductance at 18 DAT was 80.2% ($P < 0.05$) and 89.0% ($P < 0.05$) lower in the WG treatment than the control for the stress sensitive genotypes EGA Bellaroi and Tjilkuri respectively. A similar pattern was observed for stomatal

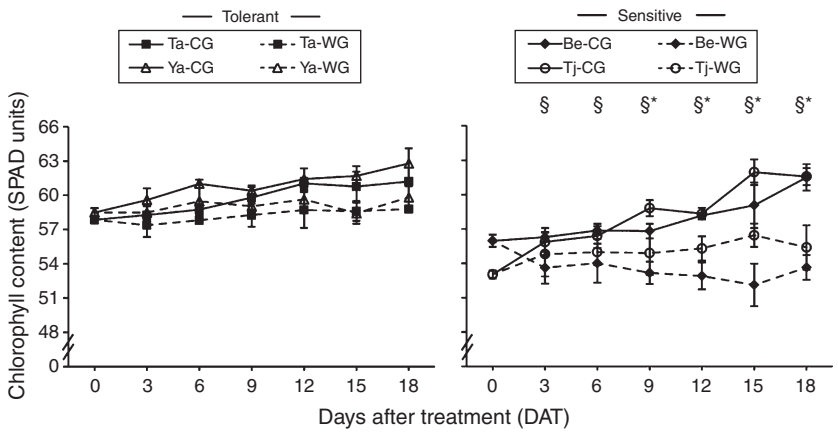

Fig. 1. Chlorophyll content (SPAD units) of four durum wheat genotypes at different time-points of pre-anthesis water-deficit stress. Abbreviations: CG, control group; WG, water-deficit stress group; Ta, Tamaroi; Ya, Yawa; Be, EGA Bellaroi; Tj, Tjilkuri. Means ± s.e. are shown for $n = 4$ at each time-point. Student's *t*-tests were performed to detect the significant differences between CG and WG in chlorophyll content for each genotype at $P < 0.05$. § indicates significant difference between CG and WG in EGA Bellaroi at that time-point; * indicates significant difference between CG and WG in Tjilkuri at that time-point.

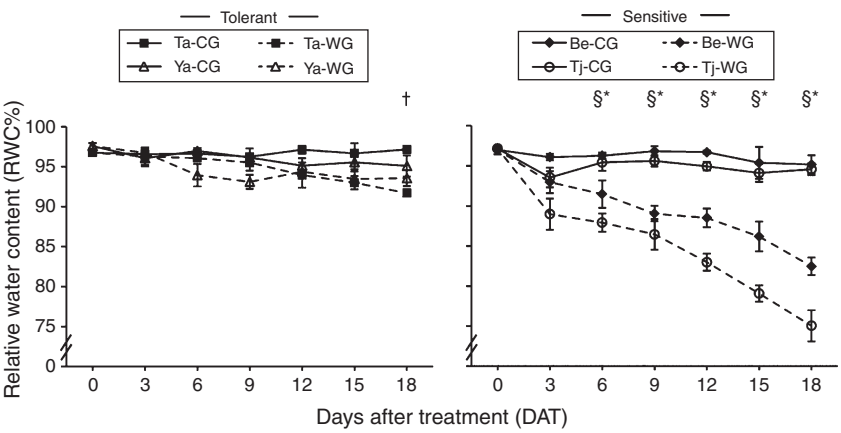

Fig. 2. Leaf relative water content (RWC%) of four durum wheat genotypes at different time-points of pre-anthesis water-deficit stress. Abbreviations: CG, control group; WG, water-deficit stress group; Ta, Tamaroi; Ya, Yawa; Be, EGA Bellaroi; Tj, Tjilkuri. Means ± s.e. are shown for $n = 3$ at each time-point. Student's *t*-tests were performed to detect the significant differences between CG and WG in RWC for each genotype at $P < 0.05$. § indicates significant difference between CG and WG in EGA Bellaroi at that time-point; * indicates significant difference between CG and WG in Tjilkuri at that time-point. † indicates significant change between CG and WG in Tamaroi at that time-point.

conductance on the abaxial surface at 18 DAT with a smaller reduction in the two tolerant genotypes in the WG treatment (86.8% ($P < 0.05$) and 81.1% ($P < 0.05$) lower than the control, Tamaroi and Yawa respectively) compared with the two sensitive genotypes (93.9% ($P < 0.05$) and 96.7% ($P < 0.05$) lower than the control, EGA Bellaroi and Tjilkuri respectively). We noted that for both abaxial and adaxial surfaces of all four genotypes, the steepest decline in stomatal conductance in WG was observed at the start of the water-deficit stress treatment (3 DAT) ($P < 0.05$). From this point onwards, for the two stress tolerant genotypes, the stomatal conductance remained stable for Yawa (no significant change observed) but increased for Tamaroi ($P < 0.05$) as the plant developed to flowering under stress. For example, the adaxial and abaxial stomatal conductance of Tamaroi stressed plants at 3 DAT was 187.0 and 34.5 mmol m^{-2} s^{-1}, whereas at 18 DAT the values increased to 296.3 ($P < 0.05$) and 59.8 ($P < 0.05$) mmol m^{-2} s^{-1} respectively. However, for the stress sensitive varieties, their disrupted stomatal conductance in the WG treatment appeared to continue to decrease, reaching almost complete stomatal closure especially on the abaxial side at 18 DAT, although significance was only detected in Tjilkuri when comparing 18

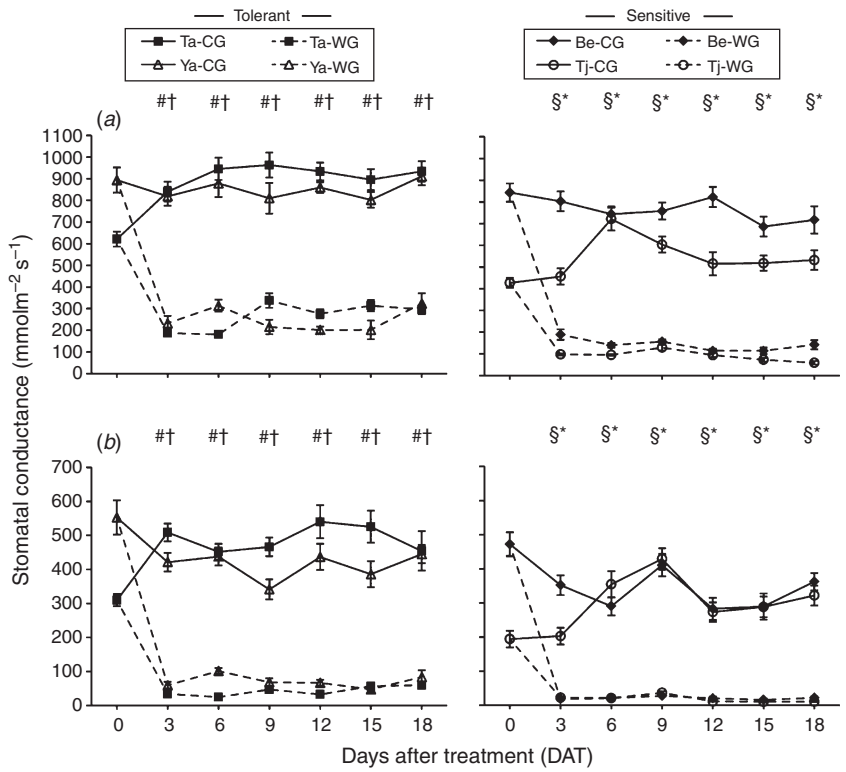

Fig. 3. Stomatal conductance (g_s, mmol m^{-2} s^{-1}) on the (*a*) adaxial leaf surface and (*b*) abaxial leaf surface of four durum wheat genotypes at different time-points of pre-anthesis water-deficit stress. Abbreviations: CG, control group; WG, water-deficit stress group; Ta, Tamaroi; Ya, Yawa; Be, EGA Bellaroi; Tj, Tjilkuri. Means ± s.e. are shown for $n = 4$ at each time-point. Student's *t*-tests were performed to detect the significant differences between CG and WG in stomatal conductance for each genotype at $P < 0.05$. § indicates significant difference between CG and WG in EGA Bellaroi at each time-point; * indicates significant difference between CG and WG in Tjilkuri at each time-point; † indicates significant difference between CG and WG in Tamaroi at each time-point. # indicates significant difference between CG and WG in Tamaroi at each time-point.

DAT to 3 DAT ($P < 0.05$). For instance, the adaxial and abaxial stomatal conductance of 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 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) and the correlation between chlorophyll content and leaf relative water content is moderate ($r = 0.50$).

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

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

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

Table 2. Effect of water-deficit stress on the morphological traits and yield components of four durum wheat genotypes

Abbreviations: CG, control group; WG, water-deficit stress group; Ta, Tamaroi; Ya, Yawa; Be, EGA Bellaroi; Tj, Tjilkuri. Means ± s.e. are shown for $n = 4$. Significance differences between the CG and WG for that genotype are indicated: *, $P < 0.05$

Genotype	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

observed in the two stress sensitive genotypes ($P < 0.05$), whereas 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 whereas 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* exhibited a trend of upregulation in response to water stress at 3, 6, 9 and 15 DAT (although not significant), with a peak of *ARF8* upregulation at 12 DAT ($P < 0.05$). A similar trend of *ARF8* upregulation was observed in the flag leaf of Yawa, where significance was detected at 6, 9 and 12 DAT ($P < 0.05$). In contrast, in the flag leaf of Tjilkuri, *ARF8* exhibited a downregulation trend under stress where significance was observed at 3, 12 and 18 DAT ($P < 0.05$). In EGA Bellaroi, significant downregulation of *ARF8* was observed at 9, 15 and 18 DAT ($P < 0.05$), where the more 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. For example, at 12 DAT, *ARF8* was significantly upregulated in Tamaroi ($P < 0.05$), but was downregulated in Yawa and Tjilkuri ($P < 0.05$). On 18 DAT, *ARF8* was significantly downregulated in the WG treatment of Tamaroi, Yawa and EGA Bellaroi ($P < 0.05$) but appears to be upregulated in Tjilkuri (although this is not significant).

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

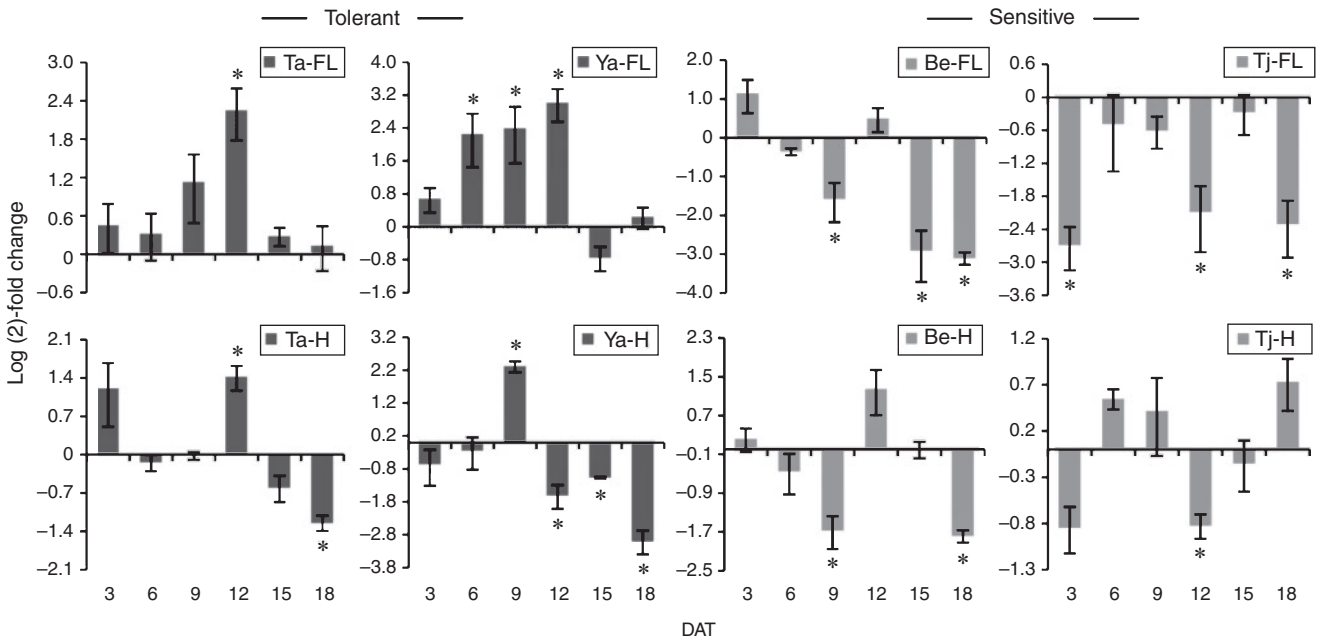

Fig. 4. Differential expression of *ARF8* in response to pre-anthesis water-deficit stress in the flag leaf and the developing head at different time-points in four durum wheat varieties. Abbreviations: DAT, days after treatment; FL, flag leaf; H, developing head; Ta, Tamaroi; Ya, Yawa; Be, EGA Bellaroi; Tj, Tjilkuri. The bars represent the log (2)-fold changes (means ± s.e. for $n = 3$) between the CG (control group) and WG (water-deficit stress group). Student's *t*-tests were performed to detect the significant differences between CG and WG in *ARF8* expression for each genotype. Significant differences ($P < 0.05$) between CG and WG at that time-point are indicated: *, $P < 0.05$.

Yawa, *ARF18* exhibited a general trend of upregulation, but with significance detected only at 15 and 18 DAT ($P < 0.05$) for Tamaroi and at 9 DAT ($P < 0.05$) for Yawa. In contrast, in the flag leaf of EGA Bellaroi and Tjilkuri, *ARF18* exhibited a general trend of downregulation, with significance detected at 12 DAT ($P < 0.05$) for EGA Bellaroi, and at 3, 6, 12 and 15 DAT ($P < 0.05$) for Tjilkuri. In the developing head tissue, the regulatory pattern of *ARF18* is subject to genotype. In Tamaroi, *ARF18* appeared to exhibit an upregulation trend but with significance detected only at 3 and 12 DAT ($P < 0.05$). In Tjilkuri, *ARF18* was significantly downregulated at 3, 6 and 15 DAT ($P < 0.05$). For Yawa and EGA Bellaroi, the regulation of *ARF18* under stress fluctuated without a clear pattern, but expression was significantly upregulated in Yawa at 12 DAT ($P < 0.05$) and downregulated in EGA Bellaroi at 12 and 18 DAT ($P < 0.05$).

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 exhibited a downregulation trend under stress, with significance detected at 3, 12, 15 and 18 DAT for Tamaroi ($P < 0.05$), and 18 DAT for Yawa ($P < 0.05$). For EGA Bellaroi and Tjilkuri, in general, upregulation of miR160 could be found with significance at 9 and 15 DAT ($P < 0.05$) for EGA Bellaroi, and 9 and 18 DAT ($P < 0.05$) 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 significantly downregulated at 15 and 18 DAT ($P < 0.05$). 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 trend in the stress tolerant varieties, upregulation trend in the stress sensitive varieties) and its targets *ARF8* and *ARF18* (upregulation trend in the stress tolerant varieties, downregulation trend 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 Tamaroi at 12 DAT between miR160 and *ARF8*).

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 focussed 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

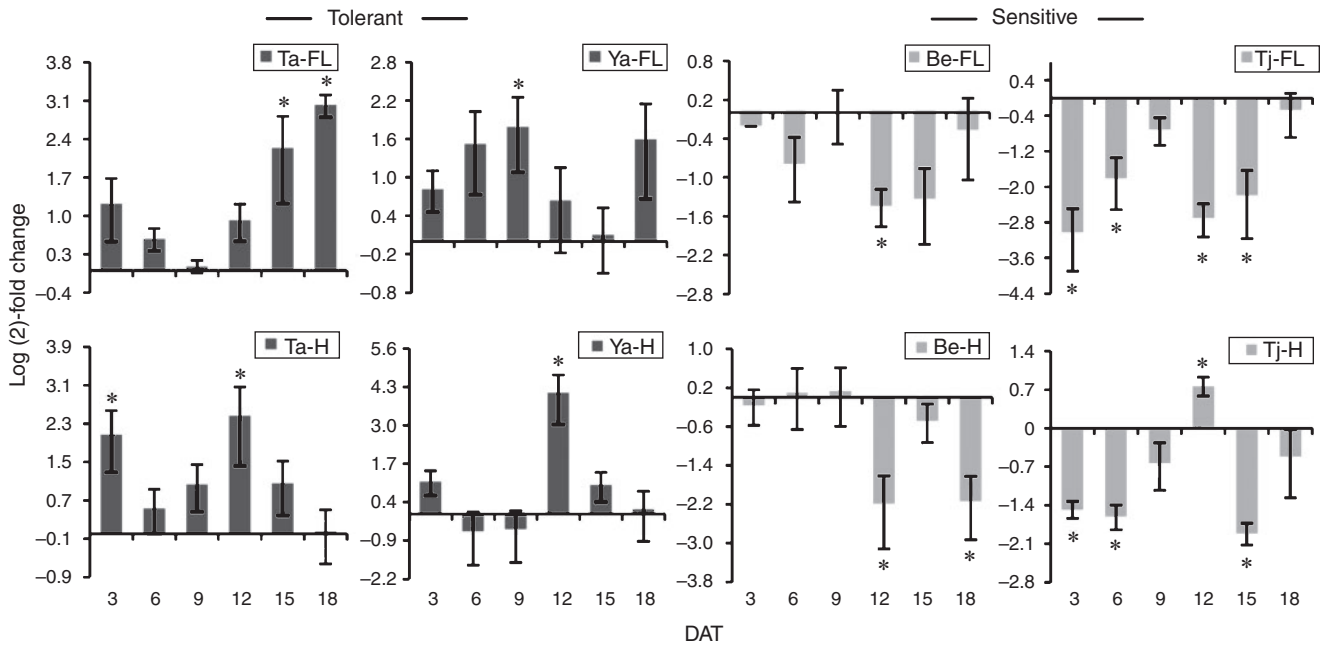

Fig. 5. Differential expression of *ARF18* in response to pre-anthesis water-deficit stress in the flag leaf and the developing head at different time-points in four durum wheat varieties. Abbreviations: DAT, days after treatment; FL, flag leaf; H, developing head; Ta, Tamaroi; Ya, Yawa; Be, EGA Bellaroi; Tj, Tjilkuri. The bars represent the log (2)-fold changes (means ± s.e. for $n = 3$) between the CG (control group) and WG (water-deficit stress group). Student's *t*-tests were performed to detect the significant differences between CG and WG in *ARF18* expression for each genotype. Significant differences ($P < 0.05$) between CG and WG at that time-point are indicated: *, $P < 0.05$.

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*. 2005; 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 in our laboratory, was selected for its potential role in stress signalling and plant development (Liu *et al*. 2016*b*). 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*. 2015*a*).

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

In the present study, distinct genotypic responses to water-deficit stress are found between the stress tolerant and sensitive durum wheat varieties across the physiological parameters measured at different time-points. 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, whereas 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

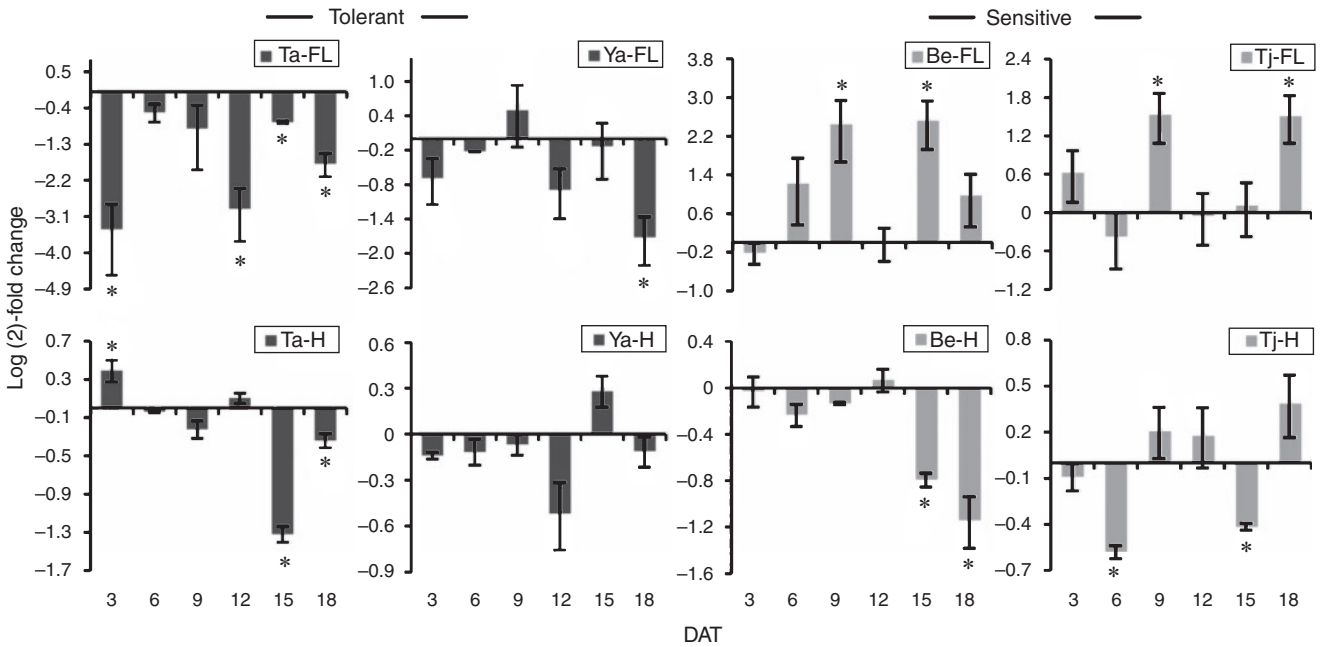

Fig. 6. Differential expression of miR160 in response to pre-anthesis water-deficit stress in the flag leaf and the developing head at different time-points in four durum wheat varieties. Abbreviations: DAT, days after treatment; FL, flag leaf; H, developing head; Ta, Tamaroi; Ya, Yawa; Be, EGA Bellaroi; Tj, Tjilkuri. The bars represent the log (2)-fold changes (means ± s.e. for $n = 3$) between the CG (control group) and WG (water-deficit stress group). Student's *t*-tests were performed to detect the significant differences between CG and WG in miR160 expression for each genotype. Significant differences ($P < 0.05$) between CG and WG at that time-point are indicated: *, $P < 0.05$.

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 notable 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 reactive oxygen species (ROS) 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*. 2006*b*). 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 co-ordinated control of the stomatal aperture. The rapid decline of stomatal conductance at 3 DAT could reduce water loss by transpiration, and 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 co-ordinated 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*. 2006*b*), 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 co-ordinated 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 co-ordinated stress signalling and adaptive physiological performance

Under environmental stress, plant developmental processes are adaptively modulated via the co-ordinated 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*. 2016*a*). 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 root development under abiotic stresses (Ding *et al*. 2008*a*; Gutierrez *et al*. 2009; Guerra *et al*. 2015; Ma *et al*. 2015). The miR167 family targets *ARF6* and *ARF8*, to regulate reproductive processes such as anther sterility and ovule development (Nagpal *et al*. 2005; Wu *et al*. 2006). In our previous study, RLM-RACE validated that durum miR160 targets both *ARF18* and *ARF8* (Liu *et al*. 2016*b*). In *Arabidopsis*, ARF18 is involved in female gametophyte and ovule development (Pagnussat *et al*. 2009; Skinner and Gasser 2009; Shi and Yang 2011) whereas in rapeseed it is associated with seed weight and silique length (Liu *et al*. 2015*c*). Additionally, all these miRNA-*ARF*s regulatory modules have complex stress responsive expression patterns under stress conditions (Jain and Khurana 2009; Tang *et al*. 2012; Liu *et al*. 2016*a*). We note that the pairing of miR160-*ARF8*/*18* also appears to be unique in durum wheat (Liu *et al*. 2016*b*) (when compared with the pairing of miR167-*ARF8* in other plant species). To further examine the interactions between miR160 and *ARF8*/*18* under stress, their expression profiles were characterised in the present study among stress tolerant and sensitive varieties at different time-points from booting to flowering.

Within each durum wheat genotype, complex temporal patterns of expression were observed for both miR160 and *ARF*s across different time-points of stress from booting to flowering. For example, in the head of Yawa, miR160 was downregulated (or unchanged) under stress at 3, 6, 9, 12 and 18 DAT, but was upregulated at 15 DAT (although not significant). In the head of EGA Bellaroi, *ARF18* did not change under stress at 3, 6 and 9 DAT, but was downregulated at 12 and 18 DAT. In addition, there was no clear negative correlation between the regulatory pattern of miR160 and *ARF8*/*18* under stress in the head tissue. Other studies which reported on the fold-changes of miRNA regulatory modules at different stages of stress treatment also identified such phenomena in expression patterns. For example, under cold stress in bread wheat during spike development, tae-miR167c was downregulated at the 1.5 and 2.0 mm anther stages, but was substantially upregulated at the 3.0 mm anther stage (meiotic division); although significant downregulation of miR167d was only found at the 1.5 mm anther stage (Tang *et al*. 2012). *ARF6* and *8*, targeted by tae-miR167 family members also had a fluctuating regulatory pattern across different stages under cold stress (Tang *et al*. 2012), but without a clear negative correlation with their miRNA. Such temporal regulatory patterns observed in the durum head tissue across different time-points of stress indicate that miRNA and ARFs could not only play a role in stress responses, but also in plant developmental processes such as anther development and fertilisation (Nagpal *et al*. 2005; 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 although 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*. 2006*a*). *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*b*; 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*) 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 transcripts of *GH3* and *IAA16*, the respective target genes of *ARF8* and *ARF18*, could not be identified in durum wheat, possibly due to the lack of full genome information. Therefore, the relationship between miRNA-mediated auxin signalling in the flag leaf, cloning of the target genes of the ARFs, its association with auxin homeostasis and cellular 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, 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 co-ordinated 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, co-ordinating 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 co-ordinated 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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