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ORIGINAL ARTICLE

microRNAs differentially modulated in response to heat and drought stress in durum wheat cultivars with contrasting water use efficiency

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Abstract Plant stress response is a complex molecular process based on transcriptional and posttranscriptional regulation of many stress-related genes. microRNAs are the best-studied class of small RNAs known to play key regulatory roles in plant response to stress, besides being involved in plant development and organogenesis. We analyzed the leaf miRNAome of two durum wheat cultivars (Cappelli and Ofanto) characterized by a contrasting water use efficiency, exposed to heat stress, and mild and severe drought stress. On the whole, we identified 98 miRNA highly similar to previously known miRNAs and grouped in 47 MIR families, as well as 85 novel candidate miRNA, putatively wheat specific. A total of 80 known and novel miRNA precursors were found differentially expressed between the two cultivars or modulated by stress and many of them showed a cultivar-specific expression profile. Interestingly, most in silico predicted targets of the miRNAs coming from the differentially expressed precursors have been experimentally linked in other species to mechanisms controlling stomatal movement, a finding in agreement with previous results showing that Cappelli has a lower stomatal conductance than Ofanto. Selected miRNAs were validated through a standardized and reliable stem-loop qRT-PCR procedure.

Keywords Drought stress · Heat stress · miRNA · Durum wheat · Stomata · WUE

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Introduction

Drought and high temperatures are among the most important environmental stresses affecting plant growth; consequently, the plants have evolved morphological and physiological adaptations, as well as molecular responses activated upon stress perception to cope with environmental constraints. The adaptation to heat and drought stress involves different strategies like shorter life cycle with accelerated flowering, reduction of water loss (i.e., stomatal closure, increased leaf cuticle thickness), and improvement of water uptake (i.e., deeper and/or larger root system). Morphological and physiological adaptations to environmental constraints are associated with wide transcriptional changes controlled by sophisticated molecular mechanisms (Cattivelli et al. 2008; Hu and Xiong 2014).

Durum wheat (*Triticum turgidum* subsp. *durum*) is an important cereal crop grown mainly in semi-arid environments, e.g., the Mediterranean regions, characterized by water scarcity and high temperatures. Consequently, drought and heat tolerance are main targets for durum breeding and many studies have been dedicated to the characterization of the physiological and molecular traits involved in the adaptation to these stress conditions. In this context, two durum wheat cultivars, Ofanto and Cappelli, have been extensively characterized since they

represent genotypes with a significantly different response to drought (De Vita et al. 2007; Rizza et al. 2012; Panio et al. 2013; Aprile et al. 2013).

In previous studies, Cappelli consistently showed a constitutive higher water use efficiency (WUE), *i.e.* the ability of a crop to produce biomass per unit of water evapotranspired, compared to Ofanto, a finding associated with a lower stomatal conductance in Cappelli vs. Ofanto during all developmental stages and over a range of relative soil water contents (Rizza et al. 2012). Cappelli represents a typical isohydric–water saver genotype, whereas Ofanto can be considered an anisohydric–water spender cultivar (Levitt 1980; Tardieu and Simonneau 1998). Indeed, Cappelli closes stomata since on incipient drought, whereas the same response in Ofanto is activated after more severe drought conditions (Rizza et al. 2012). Aprile et al. (2013) reported that Ofanto activates a large set of well-known drought-related genes after drought treatment, while Cappelli shows a minimal modulation of gene expression in the same conditions associated to the constitutive expression of a set of genes that in Ofanto are induced only upon drought stress. These findings sustain the hypothesis that Cappelli activates the onset of drought mechanisms earlier then Ofanto, when water shortage is minimal.

The burst of deep sequencing technologies in the last few years has allowed the identification and quantification of many classes of small RNAs involved in gene regulation of different biological processes among them the abiotic stress response, revealing an additional level of posttranscriptional control of gene expression (Guerra et al. 2015). miRNAs, the best characterized small RNA category, are 20–24-nt-long single-stranded RNAs, originating from longer hairpin transcripts, transcribed from specific miRNA genes (MIR genes) by RNA polymerase II. miRNAs, acting upon sequence pairing as negative posttranscriptional regulators of gene expression, have emerged as important modulators in drought and heat tolerance and avoidance via control of drought and heat responsive genes (Rogers and Chen 2013; Budak et al 2015; Carrington and Ambros 2003; Ding et al. 2013). Substantial work has been carried out to investigate miRNA expression profiles and targets during drought stress in contrasting cultivars of several crop species like cowpea (Barrera-Figueroa et al. 2011), bread wheat (Ma et al. 2015), barley (Hackenberg et al. 2015; Ferdous et al. 2016), rice (Cheah et al. 2015) and durum wheat (Liu et al. 2015, 2016).

This work aims to study the modulation of the of the durum wheat miRNAome in response to two levels of drought stress and to heat stress in two durum wheat cultivars (Cappelli and Ofanto) characterized by contrasting WUE. A total of 80 precursor miRNAs (pre-miRNAs) were found differentially expressed in at least one comparison. Several drought or heat regulated pre-miRNAs were characterized by a genotype-specific expression profile and with predicted targets mostly involved in the control of stomatal opening according to the different level of WUE of the cultivars tested.

Materials and methods

Genetic materials and growth conditions

The experiment was performed using two durum wheat (*Triticum turgidum* subsp. *durum*) cultivars, Ofanto and Cappelli. The plants were grown at 20/18 °C day/night temperature with 16/8-h photoperiod and a relative soil water content (RSWC) maintained constantly at 85 %. Stress treatments were applied when plants reached the developmental stage of fully expanded mature third leaf. As for the drought stress, water was withheld until the RSWC dropped to 55 % (mild drought stress) or to 35 % (severe water stress), and leaves were harvested 2 and 3 days, respectively, after RSWC reached the desired threshold. Developmental stage and drought stress conditions were similar to those reported by Rizza et al. (2012) where a detailed analysis of WUE was carried out using the same cultivars employed in the current work. Leaves from control plants were harvested at the same time as leaves subjected to severe stress. Heat stress consisted in 3 h at 36 °C in presence of light, and leaves were harvested immediately after. Three biological replicates for each treatment were considered; in each replicate, all leaves of five plants were pooled together and used for RNA isolation.

RNA isolation and small RNA libraries preparation

Total RNA was extracted from wheat leaves tissues using TRIzol reagent (Invitrogen) with minor modifications. RNA quality and concentration were evaluated with the Agilent 2100 Bioanalyzer RNA 6000 Nano assay. All RNA samples were stored at −80 °C until further processing. The preparation of small RNA libraries was performed with the TruSeq Small RNA Sample Prep Kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Briefly, 1 μg of total RNA was ligated with two adapters at 3′ and 5′ ends. Adapter-ligated RNA was reverse-transcribed with SuperScript II Reverse Transcriptase (Invitrogen), then PCR-amplified (15 cycles). The cDNA libraries were purified on a 6 % TBE PAGE and quality and concentration were evaluated with the Agilent 2100 Bioanalyzer DNA1000 assay. Small RNA-seq data from 24 samples (12 from cv. Cappelli and 12 from cv. Ofanto—see Table 1) were performed using a 12-plex sequencing approach with 30-nt single-end reads on GAIIx sequencer (Illumina, San Diego, CA).

Bioinformatics prediction of known and novel miRNAs

Raw sequencing data were of good quality (mean sequence Phred quality score >30), and no quality filter was applied. Sequencing reads were trimmed using the program Cutadapt (Martin 2011) version 1.8.3 with the settings: –trim-n -a TGGAATTCTC.

Table 1 List of the 24 samples used for library preparation and their acronyms

Variety	Treatment	Sample name	Acronym
Cappelli	Control	Cappelli_ctrl_1 Cappelli_ctrl_2 Cappelli_ctrl_3	CC
	Drought stress I	Cappelli_stress_lev1_rep1 Cappelli_stress_lev1_rep2 Cappelli_stress_lev_rep3	CDS I
	Drought stress II	Cappelli_stress_lev2_rep1 Cappelli_stress_lev2_rep2 Cappelli_stress_lev2_rep3	CDS II
	Heat stress	Cappelli_heat_stress_1 Cappelli_heat_stress_2 Cappelli_heat_stress_3	CHS
Ofanto	Control	Ofanto_ctrl_1 Ofanto_ctrl_2 Ofanto_ctrl_3	OC
	Drought stress I	Ofanto_stress_lev_rep1 Ofanto_stress_lev1_rep2 Ofanto_stress_lev1_rep3	ODS I
	Drought stress II	Ofanto_stress_lev2_rep1 Ofanto_stress_lev2_rep2 Ofanto_stress_lev2_rep3	ODS II
	Heat stress	Ofanto_heat_stress_1 Ofanto_heat_stress_2 Ofanto_heat_stress_3	OHS

For each sample, trimmed reads were mapped independently against the hexaploid *Triticum aestivum* cv. Chinese Spring reference genome version IWGSC2 downloaded from Ensembl Genomes (ftp://ftp.ensemblgenomes.org/pub/plants/release-26). Since the durum wheat genome is not yet available, only chromosomes belonging to the *T. aestivum* genomes A and B were considered as reference sequences to create a synthetic durum wheat reference. Mitochondrial and plastid genome were not considered in this analysis.

Bowtie (Langmead et al. 2009) version 1.0.1 was used to align trimmed reads to the reference genome allowing one mismatch. The mapping results of each sample were analyzed with ShortStack (Axtell 2013) version v. 2.0.9 with default settings. The initial annotation was refined by 183 high confident pre-miRNAs based on the current criteria for the annotation of plant miRNA (Meyers et al. 2008).

To identify conserved *miRNAs*, BLASTn (McGinnis and Madden 2004) with E-value <e−10 was applied using as subject all the hairpin sequences belonging to monocotyledonous species present in miRBase (Kozomara and Griffiths-Jones 2014) version 21. Results were manually inspected to verify the putative precursor sequences against the whole miRBase using the BLASTN function available on the website and to confirm that the most abundant duplex (5′ and 3′ miRNAs) was correctly extracted and named accordingly. Sequence abundance was normalized to Tag *per* one million (TP1M), to allow for libraries' comparisons. Finally, homeologous *miRNAs* were identified based on sequence similarity along the entire stem-loop sequence using the clustering program CD-HIT (Fu et al. 2012) with sequence identity ≥0.95.

miRNA targets

The nonredundant set of 5′ and 3′ miRNA sequences was used to predict targets in the full set of cDNA transcripts annotated in the *T. aestivum* cv. Chinese Spring version IWGSC2 downloaded from Ensembl Genomes (ftp://ftp.ensemblgenomes.org/pub/plants/release-26). The program TargetFinder (http://github.com/carringtonlab/TargetFinder); Fahlgren et al. 2007; Fahlgren and Carrington 2010) with default parameters was applied, and only results with a score cutoff ≤4 were considered as putative miRNA targets.

Gene annotation have been obtained from URGI Sequence repository (http://wheat-urgi.versailles.inra.fr/Seq-Repository/Genes-annotations), taking the version 2.2 of gene models and the human readable description (file name: ta_IWGSC_MIPSv2.2_HighConf_HUMAN_READABLE_DESCS_2014Jul18.txt) which reports the functional descriptions for all high-confidence (HCS) gene models without splice variants.

Differential expression analysis

Differentially expressed pre-miRNAs were identified using the Bioconductor R package DESeq2 (Love et al. 2014) setting FDR ≤0.1 and using Wald hypothesis test. For each MIR locus, the total read counts mapped to the hairpin sequence in each condition was considered as the input dataset to perform the expression analysis. In total, seven pairwise comparisons were performed applying a FDR-adjusted p value ≤ 0.1: (i) Ofanto (all samples) vs. Cappelli (all samples), (ii) Cappelli DSI vs. Cappelli control, (iii) Cappelli DSII vs. Cappelli control, (iv) Ofanto DSI vs. Ofanto control, (v) Ofanto DSII vs. Ofanto control, (vi) Ofanto heat stress vs. control, and (vii) Cappelli heat stress vs. control.

Validation of miRNA expression profiles via stem-loop qRT-PCR

The expression profiles of miRNAs were assayed by stem-loop quantitative real-time PCR (qRT-PCR); the primers were designed according to Varkonyi-Gasic et al. (2007) and listed in Online Resource 1. The stem-loop reverse transcriptase primer for each miRNA consisted of a selfed stem-loop sequence (GTCGTATC CAGTGCAGGGTCCGAGGTATTCGCACTGGA TACGAC) with the specificity conferred by a six

nucleotide extension at the 3′ end, which is complementary to the last six nucleotides at the 3′ end of the miRNA target. The RT reactions were performed starting from 200 ng of total RNA, using Superscript III (Invitrogen) and carried out according to the manufacturer's instructions. The reverse transcription products were amplified using a miRNA-specific forward primer and a universal reverse primer. The reactions were performed in triplicate on three independent biological replicates. The reactions were set up using SYBR Green PCR Master Mix (Life Technologies).

Different housekeeping genes, either durum wheat protein coding genes (polyubiquitin–Traes_7AL_5E8A63964 and OEP16–Traes_6AL_8262406D3.1) or small RNA nucleolar RNAs (snoRNAs), were considered for qPCR data normalization. snoRNAs sequences were taken from plant snoRNA online database (http://bioinf.scri.sari.ac.uk/cgi-bin/plant_snorna/home), and primers were designed on the loop region (carefully avoiding regions partly on the loop and partly on the stem—Online Resource 1). Ta-snoR10 showed an expression level similar to mid-expressed miRNAs and a good stability in all the conditions of the present experiment and was therefore chosen as endogenous control.

Results

Libraries statistics and analyses

A total of 24 libraries were constructed from RNA isolated from leaves of two durum wheat cultivars (Cappelli and Ofanto), subjected to two levels of drought (mild and severe) or to heat stress (Table 1). The libraries were deep sequenced with Illumina technology, and a total of 74,277,547 raw reads were generated. Out of them 59,974,281 clean reads were mapped on the A and B genomes of bread wheat considered as the putative durum wheat genome (Online Resource 2).

Small RNA size distribution for each library revealed a typical 2-peaks profile, as expected for Dicer-like protein (DCL) products (Fig. 1). Most of the libraries show the 24-nt peak higher than the 21-nt peak, as already observed for leaf-derived libraries in monocots (Ma et al. 2015; Xu et al. 2014; Wu et al. 2016; Bertolini et al. 2013), with the exception of the library from Cappelli in control conditions (CC) and of the two libraries from heat stressed plants (CHS and OHS), that show a 24-nt peak as high as the 22-nt peak. These libraries have a reduced proportion of siRNAs (typically 24 nt long), and a significant shift toward 21–22-nt molecules (typically miRNAs). miRNAs with a length of 22 nt facilitate the triggering of secondary siRNA production from their target transcripts, enhancing their silencing power (Cuperus et al. 2010; Chen et al. 2010).

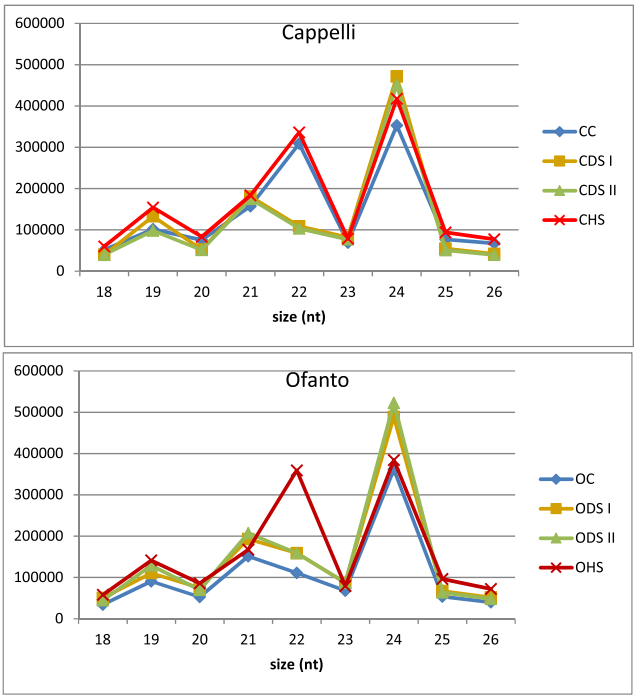


Fig. 1 Small RNA size distribution of durum wheat libraries. *X*-axis: length in nucleotides (nt); *Y*-axis: raw abundances for each size class as an average of three biological replicates, in Cappelli (*upper panel*) and in Ofanto (*lower panel*). *CC* Cappelli control, *CDSI* Cappelli drought stress I, *CDSII* Cappelli drought stress II, *CHS* Cappelli heat stress, *OC* Ofanto control, *ODSI* Ofanto drought stress I, *ODSII* Ofanto drought stress II, *OHS* Ofanto heat stress

Identification of durum wheat miRNA

The public miRNA repository miRBase (www.mirbase.org v.21) contains only one durum wheat miRNA, and few works have been published on this species (Liu et al. 2015). To advance the knowledge on miRNAs in this crop, the reads mapped to the A and B genomes of bread wheat, considered as the putative durum wheat genome, were analyzed with ShortStack pipeline (Axtell 2013) annotating a total of 37,933 clusters corresponding to genomic regions of small RNA accumulation. Successive filtering steps identified 183 high confident miRNA, matching the criteria for miRNA annotation (Meyers et al. 2008), each producing one or more miRNA duplexes. BLASTn search against miRBase database and manual refinement of in silico produced data, identified a total of 98 miRNA homologous to previously identified plants miRNAs and 85 novel miRNAs, putatively wheat specific, described hereafter as known and novel miRNAs, respectively (Online Resource 3; Fig. 2).

The set of 98 known miRNA correspond to 47 different MIR families, including 16 out of 21 highly conserved families, shown to be largely represented in almost all plant species (Cuperus et al. 2011). MIR169 (13 members), MIR156, and MIR167 (8 members) were the largest families, whereas no miRNAs were annotated as MIR408, MIR394, MIR395,

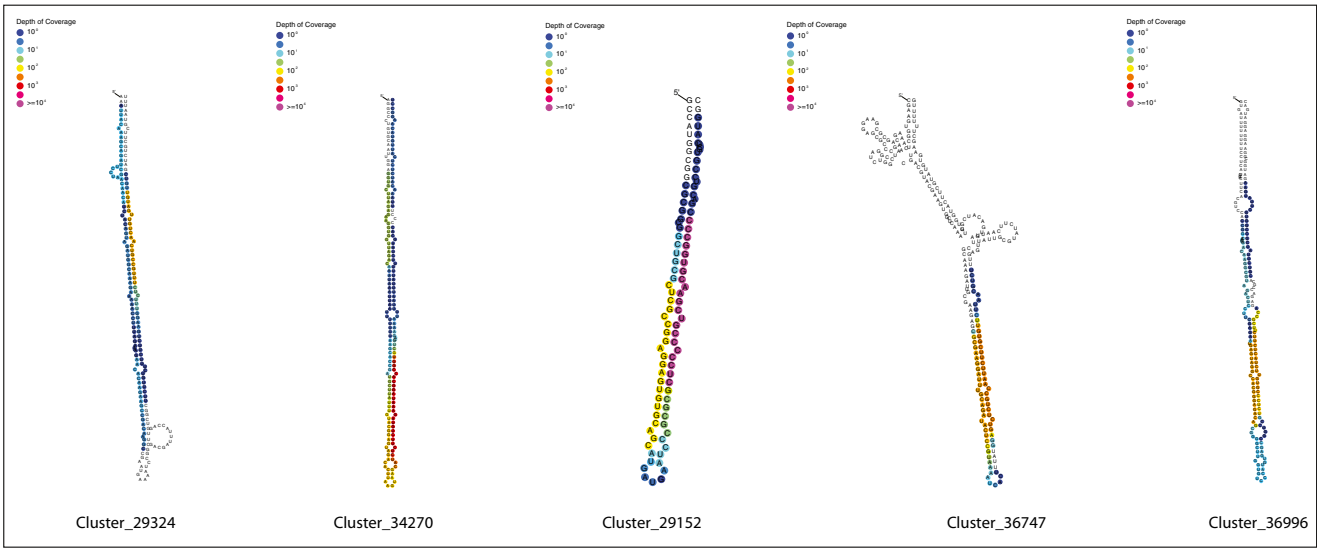


Fig. 2 Pre-miRNA secondary structure as calculated by RNAfold. Stem-loop secondary structures of five unknown miRNAs tested for by qRT-PCR (qRT-PCR data shown in Fig. 6). The color code indicates the abundance of the sequencing tag mapping to each position

MIR162, and MIR168. We annotated as 5′ and 3′ miRNAs the most abundant duplex produced by each precursor, even if sometimes it was not the most similar to previously annotated miRNAs. This is the case, for example, of Cluster_37565 and Cluster_37589, members of MIR169 family, whose most expressed miRNAs was 5 nt shifted when compared to the most canonical annotated miR169, and of Cluster_37107, whose most expressed miRNA was 2 nt shifted in respect to the annotated hvu_miR5048. In some cases, we identified the precursor as belonging to a known MIR family, even if the produced miRNA duplex is not similar with any annotated miRNA: this is the case of Cluster_16383 (miR1128) and Cluster_12532 (miR9781) that were longer than their homologous precursors, producing the most abundant duplex upstream and downstream the homology region. It should be noticed, however, that some known monocots specific families, recently identified, could have been mis-annotated. For instance, miR1122, miR1127, miR1128, miR1135, and miR1136, all belonging to the MIR1122 gene family, have been often identified only in silico, and when NGS data are available, they do not confirm previous annotations. It is not surprising, then, if the present data are not always in agreement with deposited sequences for these families.

We also identified some precursors producing phased-miRNAs, i.e., miRNAs excised from the stem of the precursor, in phase with each other (Zhang et al. 2010; Bologna et al. 2009; Addo-Quaye et al. 2009; Kurihara and Watanabe 2004). miR159 and miR169 have been already identified as miRNAs typically producing phased miRNAs (Belli Kullan et al. 2015; Contreras-Cubas et al. 2012), and here, we show three pre-miR169 producing two different duplexes (Fig. 3). ShortStack did not identified phased miRNAs for miR159 (Cluster_12004) as they were not perfect duplexes, but their abundance and the evolutionary conservation with other species sustain their existence.

Among the 85 novel miRNAs, potential candidates were grouped based on the similarity of miRNA sequences into 69 gene families, of which only 10 have more than one member.

As expected, the abundance of pre-miRNAs, summing up all the 24 libraries, is different between known and novel ones, with known pre-miRNAs far more expressed with an average expression of 236,655 TP1M against 9503 TP1M of the newly identified ones (Online Resource 4). The most expressed pre-miRNAs were Cluster_27773 (miR166), Cluster_29830 and Cluster_32083 (two members of MIR156 family), and Cluster_12004 (miR159), well-known and conserved MIR families, and Cluster_37107 (miR5048) and Cluster_24041 (miR5168), belonging to two monocots specific families, not yet well characterized.

Among unknown miRNAs, Cluster_29152 and Cluster_19417 were the most abundant precursors in the sequenced libraries with 172,629 TP1M and 136,609 TP1M, respectively. Cluster_29152, together with Cluster_4530, Cluster_8217, Cluster_27953, Cluster_13866, and Cluster_15398 are similar to miR531, although this similarity is not sufficient to define these miRNAs as putative members of this family. We also identified some miRNAs whose precursor and mature sequences were similar to two different MIR families (usually low confidence or uncharacterized MIR families). We annotated these miRNAs as novel, as their homology with already known miRNAs is not well defined. Moreover, Cluster_19417 shows a pattern of phased-small RNA production, although the phasing is not perfect and ShortStack did not select the other duplexes.

Cappelli and Ofanto were characterized by a different miRNA population even in absence of stress. Figure 4 shows

Fig. 3 MIR169 and MIR159 loci generating more than one duplex. Sequence and dot-bracket notation representing RNA stem-loop structure of some MIR loci generating more than one distinct duplex from the same precursor. For each precursor, name, MIR family, and mapping position (chromosome, coordinates, strand) are presented. *Below the dot-bracket notation*, distinct miRNA duplexes are aligned and the raw abundance (a) and length (l) of the reads are shown

the results of the comparison between the control sample of the two cultivars for those pre-miRNAs with at least a 1.5 ratio between Cappelli and Ofanto, considering the normalized abundances and summing up precursors' abundance of each MIR family (Online Resource 4). The most striking examples are Cluster_35513 and MIR5200 family nearly 15- and 10-fold, respectively, more expressed in Cappelli, while Cluster_29152 and Cluster_30941 were 80- and 13-fold, respectively, more expressed in Ofanto. Within novel miRNAs, Cluster_8217 and Cluster_15398 were undetectable in Cappelli but clearly expressed in Ofanto (about 1900 and 1300 TP1M, respectively). This analysis suggests that these novel pre-miRNAs, putatively species specific, could have an important role in varietal differences.

Genomic organization of durum wheat MIR genes

We observed an even distribution of the identified MIR loci in the A and B genomes of bread wheat considered as the putative durum wheat genome (Fig. 5), with the exception of chromosome 3B, the largest one that hosts a relative low number of predicted miRNAs (16 loci in more than 750 Mbases). Very few miRNAs are organized in tandem repeats (Online Resource 3). The most striking examples are the three members of MIR5200 family, clustered together in a 1.5-kb region on chromosome 7B. Additionally, we found the two members of MIR1432 family in 3.5 kb on chromosome 2A, two miR9674 in 1.5 kb on chromosome 4B, and two miR167 in nearly 3 kb on chromosome 5B. Two unknown and novel miRNAs are also clustered together, and based on their mature sequence, they do constitute a MIR family (Cluster_34228 and Cluster_34229, in 2 kb on chromosome 7A).

Considering the tetraploid nature of the genome of durum wheat, a specific search was dedicated to the identification of the putative homeologous miRNAs, defined as miRNA sharing more than 95 % of identity in their precursor sequences and sitting on homeologous chromosomes. We found eight couples of homeologous miRNAs: three on chromosomes 5A-5B (Cluster_24561 and Cluster_27799; Cluster_24041 and Cluster_26880; Cluster_23928 and Cluster_26932), two on chromosomes 2A-2B (Cluster_7528 and Cluster_11139; Cluster_10594 and Cluster_6194), Cluster_19682 and Cluster_21808 on chromosomes 4A-4B, Cluster_29830 and Cluster_32083 on chromosomes 6A-6B, and Cluster_34415 with Cluster_36805 on chromosomes 7A-7B.

In silico identification of target genes

We predicted the putative targets for the miRNA sequences here identified based on sequence similarity and using the program TargetFinder. Although in silico approaches can still produce large numbers of false-positive predictions, these tools are useful to describe a tentative role of newly identified and previously unknown miRNAs. When the complete set of miRNAs identified in the current work, comprising a total of 324 nonredundant miRNA sequences (considering 5′ and 3′ sequences) was used as input, a total of 257 miRNAs yielded at least one target, for a total of 2252 different targets (Online Resource 5). Conserved miRNAs usually share the same targets across different plant species (Chen 2009); indeed, most

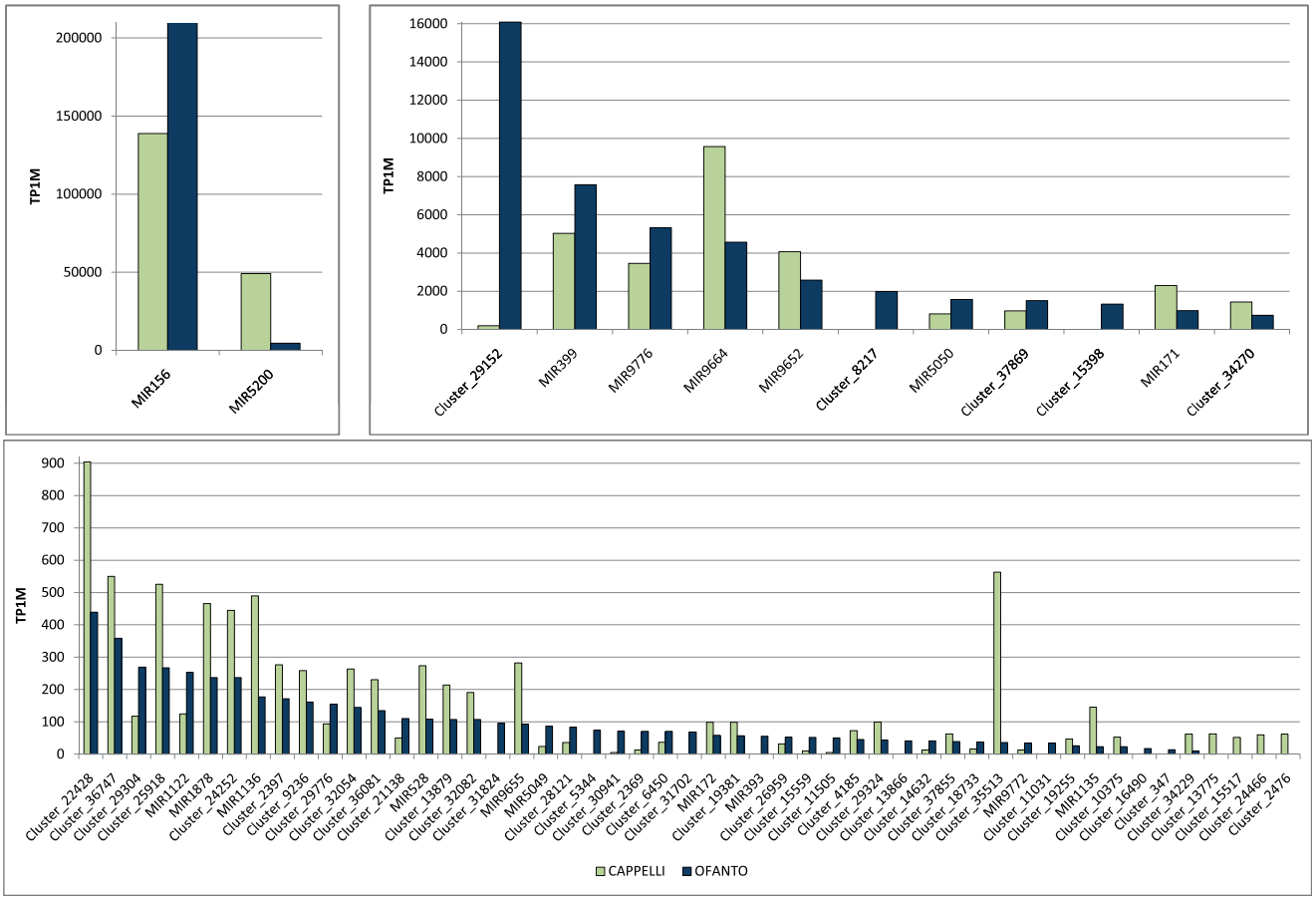


Fig. 4 Profile of pre-miRNA abundance in Ofanto and Cappelli. pre-miRNA loci normalized (TP1M) abundance in the small RNA libraries from Ofanto (*dark blue*) and Cappelli (*light green*) in control conditions have been averaged and summed up for known MIR families. Only pre-miRNA showing at least 1.5 ratio in the Ofanto vs. Cappelli comparison are shown. Pre-miRNAs have been divided in the three panels based on their abundances, to appreciate differences also among low expressed loci

target predictions for highly conserved miRNAs are as expected from predictions and validations in other species.

pre-miRNAs differentially expressed between Ofanto and Cappelli in control and drought stress conditions

The DESeq2 R/Bioconductor package (Love et al. 2014) was used to gain statistical evidence for differential expression of known and novel pre-miRNAs between Ofanto and Cappelli grown in control condition or exposed to drought stress (DSI and DSII). A total of 80 pre-miRNAs were found to be differentially regulated in at least one comparison.

The comparison Ofanto vs. Cappelli yielded a total of 44 pre-miRNAs (25 known and 19 novel) as differentially regulated, suggesting a constitutively different expression of these pre-miRNAs in the two genotypes (Table 2). Some pre-miRNAs were found more expressed in Cappelli, e.g., miR827 (Cluster_10488), miR171 (Cluster_7479), and miR5200 (Cluster_35769 and Cluster_35770), while others were more expressed in Ofanto, e.g., miR156 (Cluster_12326), miR167 (Cluster_18670), Cluster_15398, and Cluster_29152. Interestingly, the target genes of some of the miRNAs originating from these pre-miRNAs are involved in the regulation of stomatal movements. For example, the predicted target of miR5200 (Cluster_35769 and Cluster_35770) is the highly conserved florigen gene *FLOWERING LOCUS T* (*FT*), a gene also involved in the control of stomatal movement (Wu et al. 2013).

GRAS (*GIBBERELLIC–ACID INSENSITIVE* (*GAI*), *REPRESSOR of GAI* (*RGA*), *SCARECROW* (*SCR*)) proteins are plant-specific proteins with important roles in plant growth, development, phytohormone signal transduction, and stress response (Bolle 2004; Xu et al. 2015). We found *GRAS* proteins as predicted targets for miR171 from Cluster_7479, a miRNA constitutively more expressed in Cappelli compared to Ofanto (Table 2). The target of miR156 from Cluster_12326, constitutively more expressed in Ofanto vs. Cappelli, is the conserved *SQUAMOSA PROMOTER BINDING PROTEIN LIKE-PROTEIN* (*SBP*) transcription factor, which is known to be fundamental for leaf growth and development (Wu and Poethig 2006).

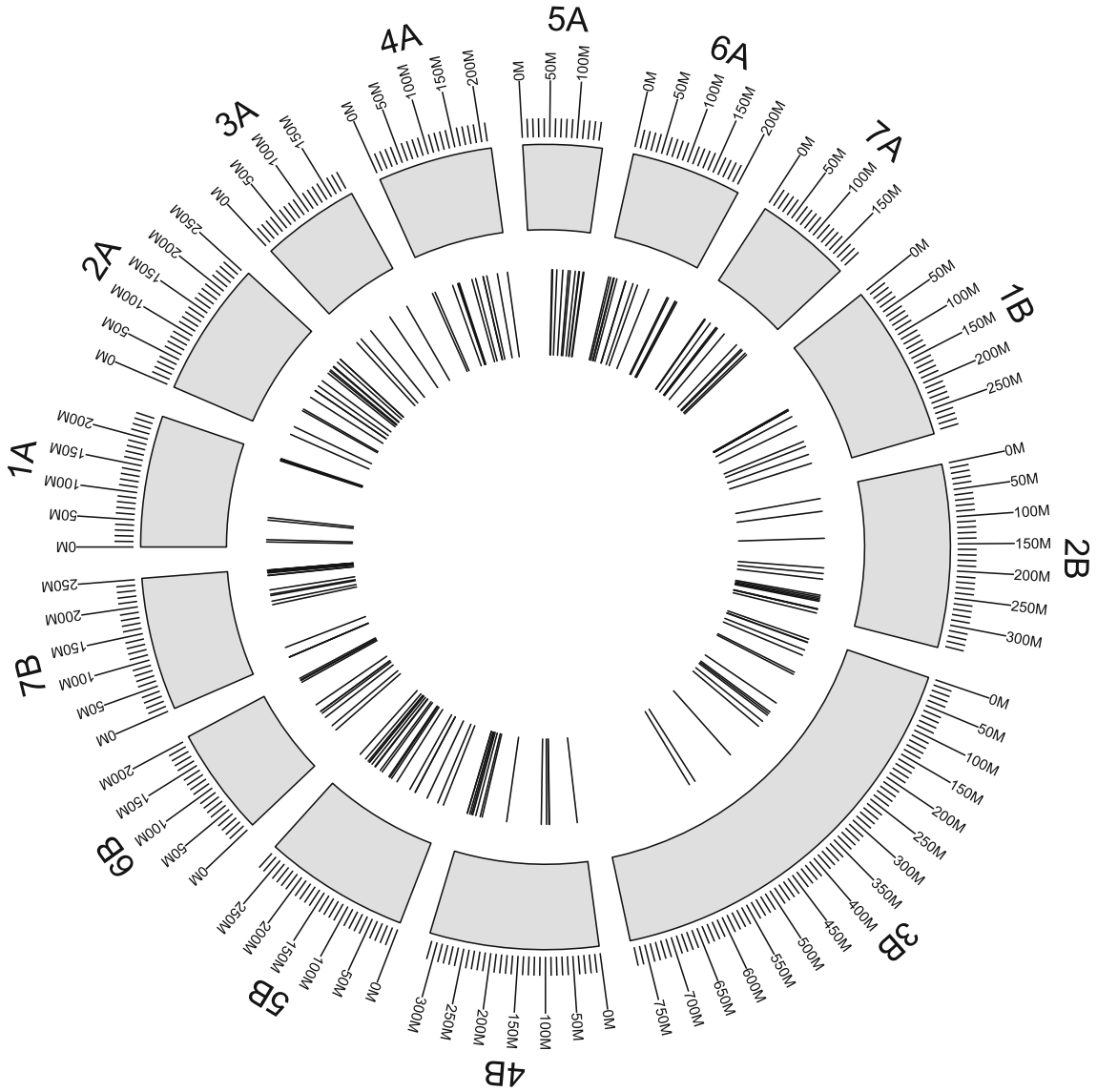


Fig. 5 Chromosome distribution of MIR loci in the *Triticum durum* synthetic genome. MIR loci, shown as *black bars*, are plotted on the circular representation of the A and B genomes of bread wheat considered as the putative durum wheat genome, based on the coordinates of the predicted pre-miRNAs

To identify drought-associated pre-miRNAs in Cappelli and Ofanto plants exposed to a mild (DSI) and to a severe (DSII) drought stress have been compared with plants grown under optimal water conditions. A total of 11 (9 known and 2 novel) and 15 pre-miRNAs (12 known and 3 novel) were found differentially expressed in Cappelli, while a total of 10 (9 known and 1 novel) and 16 pre-miRNAs (10 known and 6 novel) were found differentially expressed in Ofanto during mild and severe stress, respectively. These drought-related pre-miRNAs include sequences with the same expression profile in the two cultivars as well as sequences with a cultivar-specific expression profile.

The repression of miR169 in response to drought has been reported for several species (Khraiwesh et al. 2012; Sunkar et al. 2012; Ding et al. 2013) with a putative function in the control of stomatal movement. A strong downregulation of five family members of MIR169 has been detected in both Cappelli and Ofanto with some differences concerning their fold change and the time course expression profiles: Cluster_11103 showed a repression trend, visible already during the mild stress (DSI) in both genotypes. Cluster_21808 showed the same trend in both genotypes but higher log2 fold change in Cappelli in both DSI and DSII conditions. One of the predicted target of miR169 (Cluster_21808) is

Table 2 Differentially expressed pre-miRNAs in each comparison as evaluated by DeSeq2 (FDR ≤ 0.1)

Name	MIR family	Base mean	Log2 FC	*p* value	FDR	Name	MIR family	Base mean	Log2 FC	*p* value	FDR
OC vs. CC											
Cluster_26394	MIR1878	20.93	−0.53	0.012152431	0.05529356	Cluster_3715	MIR9664	195.32	−0.96	0.006064303	0.030658422
Cluster_35770	MIR5200	212.29	−3.52	3.97E−15	1.45E−13	Cluster_29653	MIR9670	236.96	−0.54	0.000631218	0.004595268
Cluster_11506	MIR9652	57.65	−0.80	0.001177272	0.008240902	Cluster_28123	MIR9781	21.04	1.43	4.10E−06	6.22E−05
Cluster_16383	MIR1128	21.33	−0.89	5.73E−05	0.00052107	Cluster_11505	Unknown	2.00	2.74	7.39E−06	9.61E−05
Cluster_12326	MIR156	638.18	2.32	1.73E−05	0.000196329	Cluster_13866	Unknown	1.08	3.91	0.000505542	0.003833697
Cluster_27773	MIR166	22978.08	0.54	0.02307058	0.095428309	Cluster_14632	Unknown	2.43	2.52	4.83E−05	0.000462766
Cluster_30496	MIR166	296.31	0.50	0.000335227	0.002773242	Cluster_15398	Unknown	23.48	7.98	6.07E−19	3.68E−17
Cluster_18670	MIR167	78.26	1.18	0.007695028	0.037851219	Cluster_22428	Unknown	37.76	−0.50	0.000504775	0.003833697
Cluster_32198	MIR167	773.13	−0.92	1.11E−11	3.37E−10	Cluster_2369	Unknown	2.47	2.84	1.78E−06	3.25E−05
Cluster_8221	MIR169	34.64	−1.45	0.005620928	0.029228825	Cluster_24252	Unknown	18.23	−0.63	0.00523568	0.028026287
Cluster_3862	MIR171	32.57	−0.67	0.00144881	0.009092529	Cluster_24466	Unknown	1.42	−4.70	5.23E−06	7.32E−05
Cluster_7479	MIR171	3.53	−1.11	0.009945661	0.047634483	Cluster_25918	Unknown	22.54	−0.51	0.020655356	0.089506542
Cluster_30031	MIR397	56.40	0.90	0.000174462	0.001512	Cluster_26741	Unknown	19.57	0.88	1.98E−05	0.000211517
Cluster_37696	MIR399	96.57	2.42	0.001398906	0.009092529	Cluster_29152	Unknown	434.64	5.62	1.26E−42	2.30E−40
Cluster_37107	MIR5048	2618.01	0.39	0.001905518	0.011187234	Cluster_29304	Unknown	12.60	1.22	2.34E−06	3.87E−05
Cluster_20607	MIR5050	55.84	1.21	1.96E−10	5.09E−09	Cluster_30534	Unknown	22.00	0.60	0.003771291	0.02079924
Cluster_35769	MIR5200	1312.55	−3.81	1.94E−26	1.77E−24	Cluster_31702	Unknown	1.75	5.07	2.79E−07	5.63E−06
Cluster_10488	MIR827	248.58	−1.11	0.001339232	0.009027417	Cluster_33742	Unknown	7.24	0.85	0.011581877	0.054048761
Cluster_7304	MIR827	251.34	−0.94	2.97E−05	0.000300462	Cluster_347	Unknown	1.69	2.50	0.001746456	0.010595168
Cluster_11482	MIR9652	64.26	−1.04	0.021307537	0.090185388	Cluster_35513	Unknown	12.54	−3.31	5.27E−18	2.40E−16
Cluster_29326	MIR9655	9.48	−1.09	0.002288974	0.013018542	Cluster_5344	Unknown	2.09	4.05	2.74E−07	5.63E−06
Cluster_32827	MIR9660	16.21	−1.08	1.53E−05	0.000185611	Cluster_5758	Unknown	111.20	0.50	0.014780932	0.065612916
CDS I vs CC											
Cluster_26272	MIR167	14.56	2.61	0.74810076	0.008081874	Cluster_22915	MIR399	12.64	−4.45	1.063547544	0.000938803
Cluster_10976	MIR169	398.94	−1.52	0.32688269	0.000140715	Cluster_37107	MIR5048	2249.79	0.67	0.168983209	0.002136077
Cluster_11103	MIR169	86.00	−1.38	0.258376257	0.00000563	Cluster_23249	MIR528	7.24	−1.71	0.618909856	0.075273808
Cluster_21808	MIR169	39.20	−2.35	0.406691686	0.000000968	Cluster_21463	Unknown	14.47	1.26	0.422030861	0.04235197
Cluster_8221	MIR169	50.19	−1.34	0.348803827	0.002750725	Cluster_36996	Unknown	15.43	−1.03	0.388093889	0.098378357
Cluster_23199	MIR171	37.38	2.53	0.716926566	0.007802895						

Table 2 (continued)

Name	MIR family	Base mean	Log2 FC	*p* value	FDR	Name	MIR family	Base mean	Log2 FC	*p* value	FDR
CDS II *vs.* CC											
Cluster_20013	MIR1136	5.82	−3.07	0.009953402	0.099534018	Cluster_22915	MIR399	12.64	−4.31	0.0000561	0.001402296
Cluster_34804	MIR159	315.86	2.99	2.85E−21	4.27E−19	Cluster_37107	MIR5048	2249.79	0.66	0.0000972	0.00208224
Cluster_26272	MIR167	14.56	2.51	0.000826722	0.015145312	Cluster_35769	MIR5200	2453.23	1.59	0.001333075	0.019996129
Cluster_28193	MIR167	4.69	3.60	0.000908719	0.015145312	Cluster_10488	MIR827	339.58	1.50	0.008282125	0.095836893
Cluster_10976	MIR169	398.94	−1.85	1.83E−08	0.000000916	Cluster_21463	Unknown	14.47	1.11	0.009884385	0.099534018
Cluster_11103	MIR169	86.00	−0.78	0.002151825	0.029343065	Cluster_29152	Unknown	15.43	2.28	0.00000188	0.0000705
Cluster_21808	MIR169	39.20	−2.86	1.94E−10	1.46E−08	Cluster_36996	Unknown	15.43	−1.75	0.0000353	0.001059732
Cluster_14246	MIR393	2.89	2.97	0.008305864	0.095836893						
CHS vs. CC											
Cluster_26272	MIR167	14.56	2.69	0.000346983	0.008535773	Cluster_11190	Unknown	20.03	1.56	0.004967672	0.068585457
Cluster_11103	MIR169	86.00	0.87	0.000265493	0.008163919	Cluster_29324	Unknown	9.29	1.49	0.007786405	0.095757213
Cluster_21808	MIR169	39.20	1.25	0.000259375	0.008163919	Cluster_33742	Unknown	5.13	−1.96	0.008897856	0.095757213
Cluster_28781	MIR169	22.84	2.31	0.009342167	0.095757213	Cluster_36747	Unknown	20.49	−1.10	0.005018448	0.068585457
Cluster_8221	MIR169	50.19	2.24	3.92E−14	4.82E−12	Cluster_36996	Unknown	15.43	−2.20	0.00000243	0.000149209
Cluster_23199	MIR171	37.38	2.26	0.00172194	0.035299773	Cluster_37806	Unknown	7.27	−2.95	0.004422179	0.068585457
ODS I vs. OC											
Cluster_27773	MIR166	27816.32	0.79	0.003828199	0.073161141	Cluster_28781	MIR169	24.39	−4.24	0.0000354	0.001521903
Cluster_30496	MIR166	354.57	0.59	0.002056317	0.05718974	Cluster_37589	MIR169	8.93	−2.59	0.002938914	0.063186646
Cluster_11103	MIR169	83.65	−1.30	0.00000383	0.00032966	Cluster_8221	MIR169	18.43	−1.46	0.002327489	0.05718974
Cluster_19682	MIR169	4.55	−3.37	0.001298199	0.044658039	Cluster_15262	MIR9676	57.15	0.81	0.0000108	0.000620324
Cluster_21808	MIR169	44.45	−2.32	9.48E−09	0.000001631	Cluster_36996	Unknown	25.93	−0.89	0.005123725	0.088128064
ODS II vs. OC											
Cluster_6638	MIR1432	10.27	−1.66	0.000748027	0.011540991	Cluster_24041	MIR5168	2907.45	−0.87	0.0000634	0.001800309
Cluster_34804	MIR159	496.75	3.46	2.95E−20	3.19E−18	Cluster_10488	MIR827	159.47	0.83	0.013923725	0.093985141
Cluster_30496	MIR166	354.57	0.56	0.003495309	0.034317577	Cluster_29152	Unknown	873.34	0.68	0.000159608	0.003447541
Cluster_22968	MIR167	36.41	3.31	0.005381234	0.04568823	Cluster_29324	Unknown	7.57	1.85	0.001588609	0.019063305
Cluster_10976	MIR169	345.63	−1.25	0.0000667	0.001800309	Cluster_33043	Unknown	16.46	−1.54	0.000299803	0.00539645
Cluster_11103	MIR169	83.65	−0.83	0.002239827	0.024190131	Cluster_36996	Unknown	25.93	−0.87	0.005499509	0.04568823
Cluster_21808	MIR169	44.45	−1.92	0.000000371	0.000020058	Cluster_752	Unknown	25.91	−0.64	0.010582086	0.081633234
Cluster_8221	MIR169	18.43	−1.55	0.001196765	0.016156329	Cluster_9236	Unknown	12.76	0.96	0.013275646	0.093985141

Table 2 (continued)

Name	MIR family	Base mean	Log2 FC	*p* value	FDR	Name	MIR family	Base mean	Log2 FC	*p* value	FDR
OHS vs. OC											
Cluster_21841	MIR1127	17.03	1.40	0.003816965	0.031198261	Cluster_7304	MIR827	175.53	−0.69	0.017444286	0.090893911
Cluster_12326	MIR156	1078.34	−2.55	0.001614994	0.014534942	Cluster_29024	MIR9674	1422.47	0.52	0.021983587	0.098003743
Cluster_34804	MIR159	496.75	−1.13	0.004096741	0.031198261	Cluster_11139	MIR9776	111.32	−1.08	0.0000872	0.001496756
Cluster_30496	MIR166	354.57	0.47	0.016990353	0.090893911	Cluster_7528	MIR9776	117.35	−0.75	0.000105831	0.001496756
Cluster_36963	MIR166	89.80	−1.81	0.000210114	0.002600163	Cluster_12532	MIR9781	35.71	−1.06	0.024701149	0.098003743
Cluster_18670	MIR167	111.47	−1.47	0.006068652	0.042914042	Cluster_15422	Unknown	22.99	−1.64	0.007471029	0.04930879
Cluster_26273	MIR167	1430.36	−1.14	0.0000979	0.001496756	Cluster_22428	Unknown	32.63	0.98	0.001036583	0.010262173
Cluster_32198	MIR167	543.38	−0.65	0.011322292	0.070056683	Cluster_29152	Unknown	873.34	−0.81	0.00001207	0.000298732
Cluster_21808	MIR169	44.45	1.55	0.00000105	0.0000521	Cluster_34270	Unknown	57.10	1.17	0.019682996	0.096171067
Cluster_28781	MIR169	24.39	1.98	0.024596349	0.098003743	Cluster_36747	Unknown	19.06	−1.09	0.020399923	0.096171067
Cluster_8221	MIR169	18.43	1.02	0.015956885	0.090893911	Cluster_36996	Unknown	25.93	−1.92	0.000000749	0.0000521
Cluster_24041	MIR5168	2907.45	0.99	0.0000053	0.000175043	Cluster_752	Unknown	25.91	−0.63	0.02474842	0.098003743
Cluster_35769	MIR5200	169.35	−1.25	0.000532703	0.005859734						

GLUTAMATE DECARBOXYLASE (*GAD*), an enzyme involved in the GABA biosynthesis, whose depletion affects stomatal closure and drought tolerance in *Arabidopsis* (Mekonnen et al. 2016). On the contrary, Cluster_10976 was downregulated earlier in Cappelli, while in Ofanto, was downregulated in DSII only. The predicted targets of miR169 (Cluster_10976) are different subunits of *NUCLEAR FACTOR Y, SUBUNIT A* (*NFYA*), a plant transcription factor with important roles in development and response to environmental stresses (Kumimoto et al. 2008). Another member of the same MIR family, Cluster_8221, was constitutively downregulated in Cappelli vs. Ofanto, while Cluster_28781 was strongly downregulated in Ofanto only in DSI and the predict targets are different subunit of *NFYA*. Similarly, the novel miRNA Cluster_36996 displayed a downregulation already during mild stress conditions in both genotypes with a higher log2 fold change in Cappelli than in Ofanto. Interestingly, a possible target of this candidate miRNA is *NFYA* exactly the same target of miRNA169 (Cluster_10976).

MIR159 represents another drought-related miRNA family involved in the ABA mediated pathways, and the corresponding targets are *MYB* transcription factors, positive regulators of ABA signalling (Reyes and Chua 2007). Ofanto and Cappelli showed an upregulation of Cluster_34804 (MIR159) in severe drought stress (DSII) with a log2 fold change ≥3. Likewise, Cluster_10488 and Cluster_7304 (MIR827) and the novel miRNA Cluster_29152 showed the same expression profile with a significant upregulation in DSII in both cultivars.

Among the pre-miRNAs with cultivar-specific expression profile, in Cappelli, we found, among others, miR5200 (Cluster_35769 and Cluster_35770), miR393 (Cluster_14246), miR171 (Cluster_23199), miR5048 (Cluster_ 37107), and the novel Cluster_21463 all induced, while miR399 (Cluster_22915), miR528 (Cluster_23249), and miR1136 (Cluster_20013) were repressed in response to drought stress. On the other hand, in Ofanto, miR166 (Cluster_27773) and Cluster_29324 were upregulated, while miR1432 (Cluster_6638) and Cluster_33043 were repressed. Among these, miR1432 (Cluster_6638) showed a substantial decrease in Ofanto DSII only; its downregulation under drought stress was described also in wild emmer, the ancestor of durum wheat (Kantar et al. 2011). miR1432 (Cluster_6638) is predicted to target *CALMODULIN LIKE-43*, suggesting its involvement in calcium signalling. This network is implicated in the response to abiotic stress and Ca^{2+} signals are core regulators of stomatal aperture (Dodd et al. 2010; Batistič and Kudla 2012).

Distinct members of MIR167 family, known to be regulated by drought stress also in other plant species (Ren et al. 2012; Phookaew et al. 2014), showed a peculiar expression profile in Cappelli and Ofanto. mR167 (Cluster_ 26272) was induced in both mild and severe drought stress in Cappelli only, while miR167 (Cluster_ 28193) was induced in Cappelli DSII only and miR167 (Cluster_ 22968) was upregulated after severe stress only in Ofanto. The predicted target gene of miR167 (Cluster_26272, Cluster_28193) is *ATPase FAMILY GENE 2 PROTEIN* that has been suggested to be also involved in stomatal control (Wang et al. 2013).

miRNAs differentially expressed in Ofanto and Cappelli during heat stress

The analysis of the pre-miRNAs differentially expressed in response to heat treatment have yielded a total of 12 (6 known and 6 novel) and 25 (18 known and 7 novel) pre-miRNAs in Cappelli and Ofanto, respectively (Table 2). The expression profile showed a common response for different members of MIR169 family upregulated in both genotypes, but miR169 (Cluster_8221) which is downregulated in Cappelli only. Similarly, two novel pre-miRNAs (Cluster_ 36747 and Cluster_ 36996) were downregulated after heat stress in both genotypes.

The members of MIR167 family showed a different behavior in two cultivars exposed to heat stress. The upregulation of miR167 (Cluster_ 26272) was detected in Cappelli, a trend already seen in the same cultivar subjected to drought stress. On the other hand, the downregulation of miR167 (Cluster_26273), miR167 (Cluster_32198), and miR167 (Cluster_ 2296818670) in response to heat treatment was found in Ofanto only. Overall, Ofanto downregulated more miRNA genes in response to heat stress than Cappelli, e.g., miR159 (Cluster_34804), miR166 (Cluster_36963), miR167 (Cluster_26273), miR167 (Cluster_18670), miR167 (Cluster_26272), miR9776 (Cluster_11139), miR5200 (Cluster_35769 and Cluster_35770), miR9781 (Cluster_28123), and miR159 (Cluster_34804).

Validation of differentially expressed miRNAs by stem-loop qRT-PCR

Stem-loop qRT-PCR was carried out to validate the expression profiles of ten (six known and four novel) miRNAs identified as differentially expressed after deep sequencing and selected on the basis of their expression level and the biological relevance of their putative targets (miR156/Cluster_12326, miR167/Cluster_18670, miR1432/Cluster_6638, miR159/Cluster_34804, miR169/Cluster_11103, miR169/Cluster_8221, Cluster_29324, Cluster_34270, Cluster_36747, Cluster_36996). Log2 fold changes of selected miRNAs are shown in Fig. 6. qRT-PCR produced reliable data for seven miRNAs; for these genes, the qRT-PCR analysis showed the same expression profile across all samples as Illumina deep sequence analysis. qRT-PCR analysis of the remaining three miRNAs showed few inconsistencies (one each for miR169/Cluster_11103 and Cluster_36996, and two for miR169/Cluster_8221) when compared to Illumina data (Fig. 6). Overall, the qRT-PCR analysis validates the deep sequencing data with only 4 inconsistencies over 60 comparisons.

Discussion

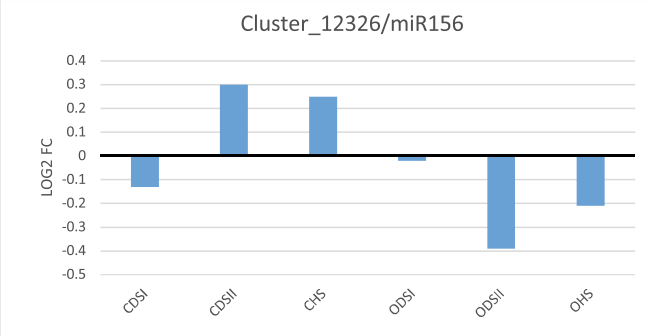
We analyzed the leaf miRNAome of two durum wheat cultivars characterized by a different water use efficiency (Rizza et al. 2012), exposed to two levels of drought stress and to heat stress. On the whole, we identified 98 miRNAs highly similar to previously known miRNAs and grouped in 47 MIR families, as well as 85 novel candidate miRNA, putatively wheat specific. We found some minor variations in length or nucleotidic sequence in respect to known monocots miRNAs for most predictions, with the exception of few miR169 precursors and members of MIR9781 and MIR1122 families, which could have been mis-annotated in *T. aestivum*.

Notably, we did not identified five highly conserved MIR families. MIR408 and MIR162 did not have almost any sequencing tags, suggesting their absence in the leaf of young durum wheat plants. A similar result was reported for miR162 in *Brachypodium* young leaves in a previous work (Bertolini et al. 2013). MIR394, MIR395, and MIR168 were present in our libraries, but were unable to pass the stringent parameters of the ShortStack pipeline.

Considering the 85 novel miRNAs identified, they are, on average, less expressed than known miRNAs, as expected, and rarely grouped into families. The main exception is the case of the six novel miRNAs similar to MIR531 family (Cluster_29152, Cluster_4530, Cluster_8217, Cluster_27953, Cluster_13866, Cluster_15398), but not enough to be considered real members of this family.

Ofanto and Cappelli, two durum wheat cultivars with different miRNAomes

This work is dedicated to the analysis of two durum wheat cultivars well characterized at physiological level for their different WUE, largely due to the different stomatal conductance (Rizza et al. 2012). It is known that plants may have different physiological behaviors with respect to water fluxes with important consequences for their survival, growth, and yield. Cappelli, a typical old cultivar, displays a water conserving behavior (isohydric plant); on the contrary, a modern cultivar such as Ofanto, displays a risk-taking behavior (anisohydric plants). Isohydric plants maintain a relatively constant leaf relative water content by reducing evaporation when facing with


Cluster_12326/miR156
LOG2 FC
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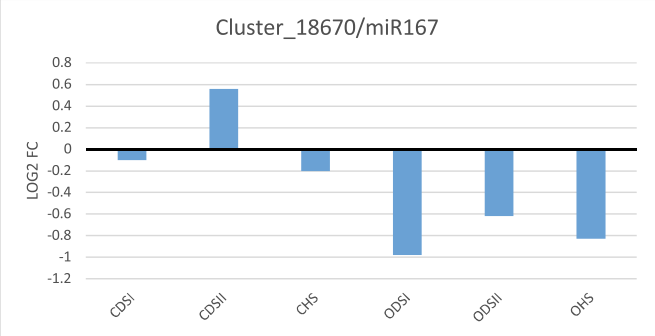
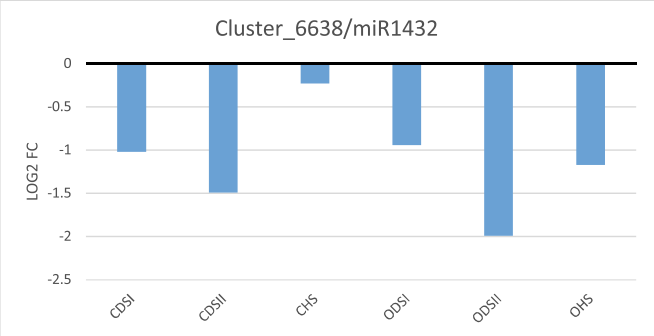
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
CDSI
CDSII
CHS
ODSI
ODSII
OHS


Cluster_18670/miR167
LOG2 FC
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
CDSI
CDSII
CHS
ODSI
ODSII
OHS


Cluster_6638/miR1432
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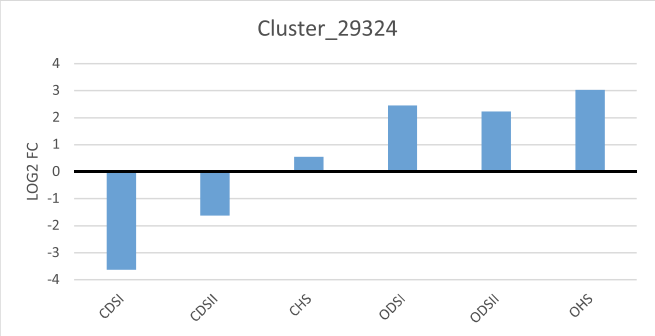
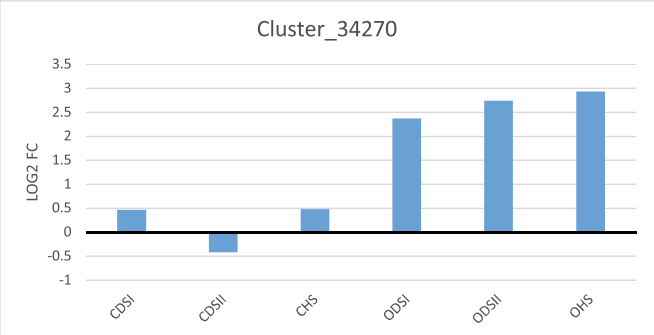
-0.5
-1
-1.5
-2
-2.5
CDSI
CDSII
CHS
ODSI
ODSII
OHS


Cluster_29324
LOG2 FC
4
3
2
1
0
-1
-2
-3
-4
CDSI
CDSII
CHS
ODSI
ODSII
OHS


Cluster_34270
LOG2 FC
3.5
3
2.5
2
1.5
1
0.5
0
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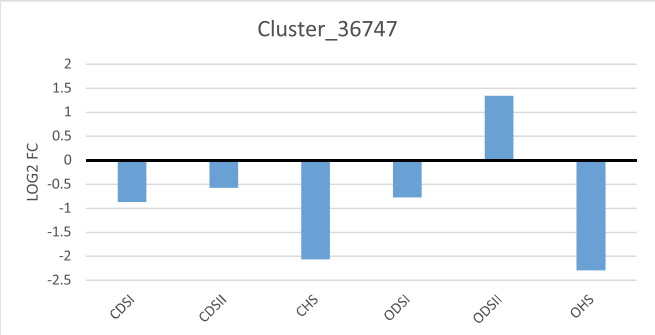
-1
CDSI
CDSII
CHS
ODSI
ODSII
OHS


Cluster_36747
LOG2 FC
2
1.5
1
0.5
0
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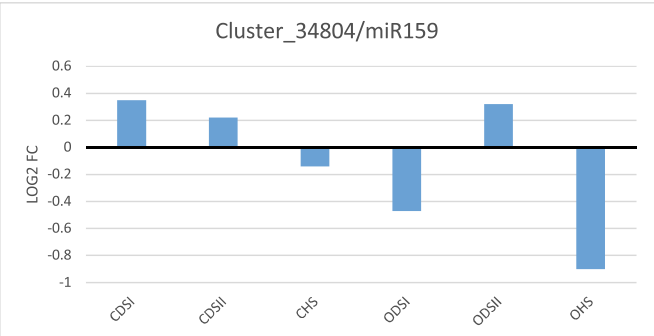
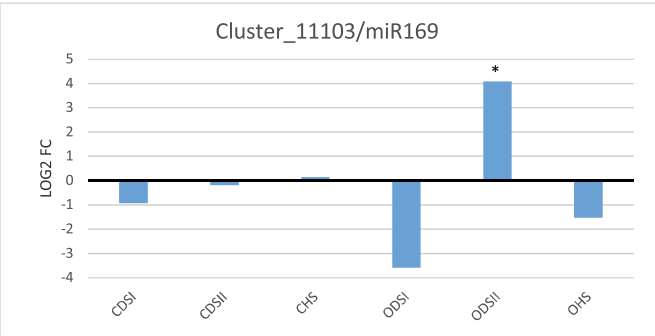
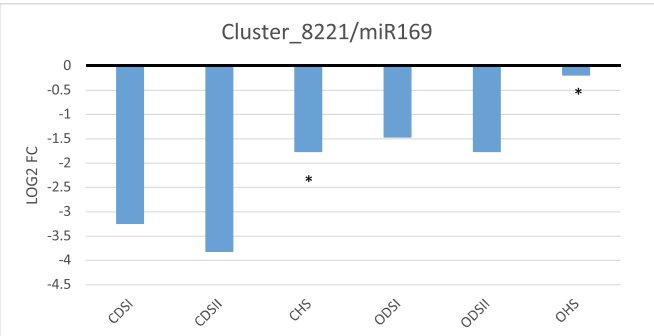
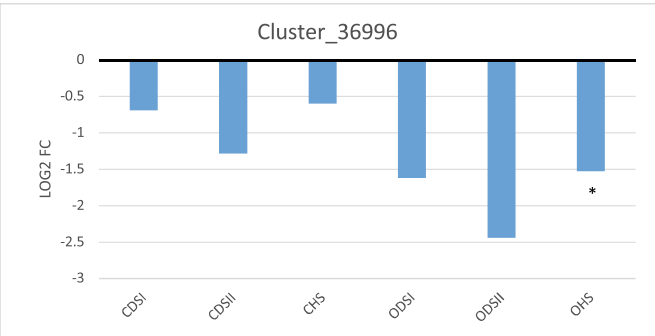
-1
-1.5
-2
-2.5
CDSI
CDSII
CHS
ODSI
ODSII
OHS


Cluster_34804/miR159
LOG2 FC
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
CDSI
CDSII
CHS
ODSI
ODSII
OHS


Cluster_11103/miR169
LOG2 FC
5
4
3
2
1
0
-1
-2
-3
-4
*
CDSI
CDSII
CHS
ODSI
ODSII
OHS


Cluster_8221/miR169
LOG2 FC
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
*
*
CDSI
CDSII
CHS
ODSI
ODSII
OHS


Cluster_36996
LOG2 FC
0
-0.5
-1
-1.5
-2
-2.5
-3
*
CDSI
CDSII
CHS
ODSI
ODSII
OHS

◀ **Fig. 6** qRT-PCR expression analysis of selected stress responsive miRNAs. Log2 fold change (FC) indicates the miRNA expression in the different comparisons: Cappelli drought stress I (CDSI) vs. Cappelli control (CC); Cappelli drought stress II (CDSII) vs. CC; Cappelli heat stress (CHS) vs. CC; Ofanto drought stress I (ODSI) vs. Ofanto control (OC); Ofanto drought stress II (ODSII) vs. OC; Ofanto heat stress (OHS) vs. OC. The wheat snoR10 was used as an endogenous control. *Asterisk* indicates the samples with different expression trend between Illumina deep sequence and qRT-PCR data

water stress. Anisohydric plants allow leaf water potential to decrease with rising evaporative demand, reaching a lower relative water content under drought conditions compared to situations in which they are well-watered (Tardieu and Simonneau 1998). Stomatal control is the key for the regulation of evapotraspiration; nevertheless, there is limited knowledge of the molecular and cellular criteria differentiating these two types of plants, which constrains our ability to manipulate the stomata behavior of crop species to improve either water use efficiency or drought tolerance (Moshelion et al. 2015).

We initially analyzed the varietal difference between Ofanto and Cappelli comparing the relative pre-miRNA expression. Differential expression levels are evident for genes such as Cluster_35770 and Cluster_35769 (MIR5200) far more expressed in Cappelli than in Ofanto, a precursor whose mature has *FT* as predicted target. Similarly, many novel pre-miRNAs were also strongly modulated in Cappelli vs. Ofanto or were shut down in one of the two cultivars (Table 2, Fig. 4). This is the case, for example of Cluster_15398 and Cluster_29152, more expressed in Ofanto, and of Cluster_35513 and Cluster_24466 much more expressed in Cappelli. This particular feature suggests that novel and putatively wheat specific miRNAs are involved in the definition of varietal differences. A deeper understanding of the functional role of such miRNAs is instrumental to further characterize the small RNA–driven differences between these two contrasting genotypes.

A comparison of the miRNAs identified as responsive to drought in durum wheat leaves with those described in response to the same stress in durum wheat and wild emmer root tissues identified only two miRNAs with a similar expression profile (miR169 and miR1136, Akpinar et al. 2015), suggesting that variation in the miRNAome in response to stress are largely tissue specific. Notably, the miR1136 in both experiments has been found downregulated only in the tolerant genotypes.

A total of 49 pre-miRNAs (known and novel) were found differentially expressed in Ofanto (37) and Cappelli (24) during drought and heat stresses. Some of them were part of a common response to stress, while for most of them, we have highlighted a cultivar specific expression profiles. A strong upregulation of miR159 in response to drought is known in the literature (Reyes and Chua 2007), and it was confirmed in both cultivars. This miRNA targets, in durum wheat also, *MYB33* and *MYB65*, two transcription factors of the ABA signalling pathway. miR159 overexpression in *Arabidopsis* suppresses *MYB33* transcript level making plants hyposensitive to ABA and desensitize hormone signalling during seedling stress responses (Reyes and Chua 2007).

Our work highlights that many of the differences in miRNA expression between the two durum wheat cultivars with contrasting stomatal conductance and exposed to drought stress have as putative target genes that in other species have been experimentally linked to stomatal controlling mechanisms. MIR169 is one of the largest and conserved MIR families; its involvement in drought stress is reported for several species although with contrasting expression patterns. For example, two members of the MIR169 family in rice, miR169g and miR169n/o, and one member in tomato, miR169c, were upregulated by drought stress. In addition, overexpression of miR169c in tomato reduced stomatal conductance and water loss showing enhanced drought tolerance in transgenic tomato lines (Zhang et al. 2011; Zhao et al. 2009). Conversely, the repression of MIR169 family members has been reported for several species and also associated to drought stress response (Khraiwesh et al. 20122012; Sunkar et al. 2012; Ding et al. 2013). In *Arabidopsis*, the downregulation of miR169a, which targets the *NFYA5*, contributes to the high level of *NFYA5* expression detected in response to drought and ABA (Kumimoto et al. 2008). *NFYA5* was found highly expressed in vascular tissues and guard cells, and the analysis of *nfya5* knockout plants and of miR169a or *NFYA5* overexpression lines showed that *NFYA5* has a role in the stomatal control and, hence, drought resistance (Li et al. 2008). In durum wheat, we found a downregulation, more in Cappelli than in Ofanto, of some members of MIR169 family having *NFYA5* has putative target. Furthermore, the novel miRNA Cluster_36996, targeting the same *NFYA* as miR169 (Cluster_10976), follows the same expression pattern as miR169 being more downregulated in Cappelli than in Ofanto in response to drought. One of the predicted targets of miR169 (Cluster_21808), more downregulated in Cappelli than in Ofanto, is glutamate decarboxylase (GAD), a key enzyme of the gamma-aminobutyric acid (GABA) biosynthesis. A rapid accumulation of GABA during abiotic and biotic stresses is well documented. Mekonnen and collaborators (2016) found that *gad1/2* mutant of *Arabidopsis* wilted earlier than wild type when exposed to drought stress due to increased stomatal aperture and to a deficit in stomatal closure, driven by a reduced GABA accumulation.

One of the predicted targets of the novel Cluster_15398, constitutively more expressed in Ofanto than in Cappelli, is an *ATP-BINDING CASSETTE* (*ABC*) *TRANSPORTER B FAMILY* member. *ABC* proteins are key players of cellular processes involved in auxin transport, lipid catabolism, xenobiotic detoxification, disease resistance, and stomatal function (Cho and Cho 2013). Interestingly, *ABCB14* has

been known as a malate importer modulating stomatal movement in guard cells (Lee et al. 2008).

miR5200 (Cluster_35769 and Cluster_35770), constitutively more expressed in Cappelli and further upregulated under severe drought stress in the same cultivar, targets *FT*. Evidences in the literature demonstrated that miR5200 directly mediate *FT* post transcriptional modulation in *Pooideae* plants (Wu et al. 2013), and it is known that *FT* is expressed in guard cells and regulates stomatal opening. Accordingly, transgenic plants overexpressing *FT* in guard cell showed open stomata, while a loss of function FT allele exhibited closed stomata (Kinoshita et al. 2011).

miR393 is specifically upregulated in Cappelli exposed to severe drought stress. Transgenic overexpression of miR393 in rice resulted in hyposensitivity to synthetic auxin analogue treatments (Xia et al. 2012), suggesting that miR393 may regulate auxin signalling and would thus reduce plant growth under drought stress. miR393 was found to target *TRANSPORT INHIBITOR RESPONSE 1* (*TIR1*), known as an auxin receptor and positive regulator of auxin signaling that acts via degradation of Aux/IAA proteins (Dharmasiri and Estelle 2002; Windels and Vazquez 2011).

Additional pre-miRNAs with putative targets that in other species have been experimentally linked to stomatal regulation are miR1432 (Cluster_6638) and miR167. The first one (downregulated in Ofanto), has *CALMODULIN LIKE-43* as putative target, a gene known for its involvement in the response of stomata to ABA (De Silva et al. 1985). miR167 (Cluster_26272, Cluster_28193), a sequence upregulated in Cappelli in response to drought treatments, has an *ATPASE FAMILY GENE 2 PROTEIN* as putative target. H^+*-ATPases* are known to be responsible for the hyperpolarization of the plasma membranes and their inhibition is essential to induce ABA mediated stomatal closure (Merlot et al. 2007). Blue light activated H^+*-ATPase* induces hyperpolarization of the plasma membrane which allows K^+ uptake which, in turn, leads the swelling of guard cells and stomatal opening. Noteworthy, *FT* is suggested to be a positive regulator for stomatal opening via its effect on the activation status of the plasma membrane H^+*-ATPase* (Kinoshita et al. 2011). Overexpression of plasma membrane H^+*-ATPase* in guard cells promotes light-induced stomatal opening and enhances plant growth (Wang et al. 2013).

The analysis of the pre-miRNAs differentially expressed in response to heat treatment has led to 12 and 25 miRNA in Cappelli and Ofanto, respectively. Noteworthy, most of the miRNAs modulated in Ofanto were downregulated (15 out of 25), while in Cappelli, only three miRNAs showed the same expression trend. miR159 is the best known heat-responsive miRNA. Its putative target genes were identified as *MYB* domain protein (*MYB33*, *MYB65*). *OsMYB55* can enhance the vegetative growth and improve grain yield of rice growth under high temperature conditions increasing the level of total amino acids (glutamine acid, proline, arginine, and GABA) (El-kereamy et al. 2012). miR159 was also shown to regulate *TaGAMYB* in bread wheat and rice transgenic lines overexpressing wheat precursor of miR159 showed a loss of heat tolerance suggesting its involvement in a heat stress-related signaling pathway. Interestingly, the *TaGAMYB1* homologous *Arabidopsis* genes (*AtMYB33* and *AtMYB65*) are also heat inducible (Wang et al. 2012). In the Cappelli-Ofanto experimental system, we confirmed the downregulation of miR159 (Cluster_34804) during heat treatment in Ofanto only, a finding that might suggest a minor heat sensitivity in this modern cultivar.

Transcriptomic analyses have shown that Ofanto activates a large set of genes in response to drought and heat stress, while Cappelli is characterized by the constitutive expression of several stress-related genes that in Ofanto are regulated only upon stress treatment (Aprile et al. 2013). This trend is confirmed also under the miRNA perspective, in particular during the heat stress response.

Overall, the data here presented indicate that Cappelli and Ofanto are characterized by significantly different miRNAomes and many of the differences in pre-miRNA expression in response to drought stress can be putatively linked to the stomatal controlling mechanisms. This finding matches the different stomatal conductance that was found when the same cultivars were investigated at physiological level (Rizza et al. 2012). Taken together, these studies suggest that the old cultivar Cappelli has a more conservative behavior with several stress-related mechanisms constitutively activated. On the contrary, the modern cultivar Ofanto represents a typical risk-taken plant and under conditions characterized by adequate irrigation and mild to moderate abiotic stress, this strategy proves advantageous, and risk-taken plants may outperform isohydric plants in terms of growth and yield (Moshelion et al. 2015).

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Author contributions LG conducted the wet lab work and data analysis. EM interpreted the bioinformatics data and wrote the manuscript. EB designed and carried out all the computational analyses for microRNA identification and differential expression analysis. AMDL prepared the plants and RNA samples. PF contributed to bioinformatics analysis. LC designed the study, contributed to the development of the project, and edited the manuscript. CC conducted the wet lab work and wrote the manuscript. All authors have read, edited, and approved the manuscript.

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