



Elucidating the regulatory roles of microRNAs in maize (*Zea mays* L.) leaf growth response to chilling stress

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Abstract

Main conclusion miRNAs control leaf size of maize crop during chilling stress tolerance by regulating developmentally important transcriptional factors and sustaining redox homeostasis of cells.

Abstract Chilling temperature (0–15 °C) is a major constraint for the cultivation of maize (*Zea mays*) which inhibits the early growth of maize leading to reduction in leaf size. Growth and development take place in meristem, elongation, and mature zones that are linearly located along the leaf base to tip. To prevent shortening of leaf caused by chilling, this study aims to elucidate the regulatory roles of microRNA (miRNA) genes in the controlling process switching between growth and developmental stages. In this respect, hybrid maize ADA313 seedlings were treated to the chilling temperature which caused 26% and 29% reduction in the final leaf length and a decline in cell production of the fourth leaf. The flow cytometry data integrated with the expression analysis of cell cycle genes indicated that the reason for the decline was a failure proceeding from G2/M rather than G1/S. Through an miRNome analysis of 321 known maize miRNAs, 24, 6, and 20 miRNAs were assigned to putative meristem, elongation, and mature zones, respectively according to their chilling response. To gain deeper insight into decreased cell production, in silico, target prediction analysis was performed for meristem specific miRNAs. Among the miRNAs, miR160, miR319, miR395, miR396, miR408, miR528, and miR1432 were selected for confirming the potential of negative regulation with their predicted targets by qRT-PCR. These findings indicated evidence for improvement of growth and yield under chilling stress of the maize.

Keywords Chilling tolerance · Leaf size · Maize microRNAs · miRNome · Organ growth · Plant cell cycle

Abbreviations

LER	Leaf elongation rate
P	Cell production
T _c	Cell cycle duration
CYC	Cyclin
TCPs	TEOSINTE BRANCHED1, CYCLOIDEA and PCF coding gene like transcription factors
GAMYB	Gibberellin- And Abscisic Acid-Regulated MYB
ARFs	Auxin responsive factor transcription factors
GRFs	growth-regulating factors

VEP1	(3-Oxo-Delta(45)-steroid 5-beta-reductase
SOD	Superoxide dismutase
WAK3	Wall-associated kinase 3
CMT2	TRNA (cytosine(34)-C(5))-methyltransferase-like isoform X2

Introduction

Maize (*Zea mays* L.), being a tropical crop plant, is very sensitive to temperatures below 17 °C which cause chilling stress (0–15 °C) resulting in growth retardation and yield loss (Rymen et al. 2007). Improved chilling tolerance would allow earlier sowing dates aiming to prevent summer drought, pests, and diseases (Kucharik 2006). Earlier sowing could also supply longer vegetation periods, which allow higher biomass accumulation. Chilling tolerant maize varieties would be advantageous for Northern countries that experience cool and short summers. Chilling stress affects

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all maize developmental stages, but maize seedlings are specifically sensitive to chilling stress observed during the transition from heterotrophic to autotrophic growth. Therefore, elucidating the mechanism of maize chilling tolerance at the seedling stage would help genetic improvement of this trait (Sobkowiak et al. 2016).

Chilling stress affects photosynthesis, root function, water relations, and transport processes through disrupting membrane integrity (Janowiak and Markowski 1994; Sowinski 1995; Richner et al. 1996; Thomashow 1999). As a result of all these impacts, organ size is reduced by inhibition of growth mechanisms such as cell division and cell expansion (Sprangers et al. 2016). The size of the leaf, which influences photosynthesis capacity, is crucial for biomass accumulation. Plant cells develop tolerance to chilling by altering their membrane lipid composition, activating reactive oxygen species (ROS) scavenging systems, proline accumulation, regulating gene expression, and biochemical pathways, all of which influence photosynthetic activity. Recent genome-wide transcriptomics analyses on maize showed that low temperature triggered the genes related to cytokinin glucoside biosynthesis, transcription factors, kinases, and phosphatases, stress response components such as drought, heat, light, wounding, oxidative, aluminum, and pathogens and ROS scavengers, such as ascorbate peroxidases, monodehydroascorbate reductases and glutathione synthetase, circadian clock, carbohydrate metabolism, cell wall modification, and cell-to-cell transport (Sobkowiak et al. 2014, 2016; Avilla et al. 2018). All these stress-responsive genes are reprogrammed by transcriptionally, post-transcriptionally, and translationally to maintain cellular function (Van Buskirk and Thomashow 2006). Among the key players in the regulation of gene expression in plants are microRNAs (miRNAs), which are endogenous, small non-coding RNAs repress the expression of target genes by base-pairing at the post-transcriptional and transcriptional levels (Bartel 2004; Wang et al. 2019).

Since the discovery of plant miRNAs in 2002, they have been shown to act as regulators in many biological processes by advanced bioinformatics tools, microarray analysis, and next-generation deep sequencing methods (Llave et al. 2002; Rhoades et al. 2002; Reinhart et al. 2002; Mallory and Vaucheret 2006; Liu et al. 2008; Li and Ruan 2009; Lv et al. 2010; Aydinoglu and Lucas 2019). As miRNAs target more than one gene at one time, miRNA-mediated post-transcriptional regulation is regarded as one of the most promising technologies for improvement of polygenic traits such as increasing of stress resistance, crop yield, and biomass production (Khraiwesh et al. 2012; Sun 2012; Zhang et al. 2014; Shriram et al. 2016). Many studies on several plant species have shed light on the miRNA-based regulation of gene expression under low-temperature stress and suggested that miRNAs can be used in applications for

improving stress tolerance of crop plants (Aydinoglu and Aktug 2017; Megha et al. 2018).

The role of miRNAs in response to cold stress was first identified for the Arabidopsis where miR319c, miR393, and miR398a were shown to be differentially regulated after chilling (Sunkar and Zhu 2004; Sunkar et al. 2006). Subsequently, the miRNA families including miR156, miR164, miR166, miR167, miR171, miR394, miR395, miR396, miR397, and miR408 have been analyzed in cold stress response in various plant species, where they target genes involved in signalling pathways and synthesis of cold stress response proteins (Xu et al. 2016; Megha et al. 2018). However, based on several observations, the response of a particular miRNA to the same stress level might vary depending on the differences in plant species. The miRNA expression levels can vary greatly depending on species, genotype, or tissue type, and members within the same miRNA family may perform different functions. Therefore, specific functional studies are necessary for each crop of interest.

Despite there is evidence on miRNAs in relation to chilling stress in various plant species, there are no functional studies showing the role of miRNAs in chilling tolerance of maize during leaf growth and development. Leaf size depends on cell number and cell size that are determined by cell division and cell expansion that take place in discrete growth zones (meristem and elongation zones). The elucidation of miRNA activity in these zones during chilling stress will contribute to our understanding of stress tolerance mechanism aiming to keep leaf size unchanged. Therefore, this study focuses on the relationships between miRNAs and their targets during growth of maize hybrid ADA313 under chilling stress through morphological observation, cellular and transcriptional analysis in the meristem, elongation, and mature zones. The results will provide insights into the regulatory roles of miRNAs under chilling stress in maize leaf growth and development.

Materials and methods

Plant material and growth conditions

All analysis was performed using the maize (*Zea mays*) hybrid ADA313 which was developed by the Maize Research Institute (Sakarya, Turkey) for Turkish agriculture. Maize seeds were sown into 1.5 l pots and placed in a growth chamber for germination. Control seedlings were grown under a 16 h day/8 h night photoperiod, 25 °C/20 °C day/night temperature, 15,000 lx light intensity, and 70% relative humidity. For chilling stress treatment, night temperature was lowered to 4 °C, while other conditions were kept unchanged. Chilling treatment was applied after germination of seeds until the fourth leaf fully got matured. The fourth

leaf of each seedling was used in all analysis. It was harvested 48 h after its appearance at the end of the night period as soon as the lights turned on. Content of photosynthetic pigments was determined according to the method and equations presented in Lichtenthaler and Buschmann (2001).

Kinematic analysis of leaf growth

The kinematic analysis was performed to determine growth parameters, as shown in Rymen et al. (2010). Briefly, the fourth leaf (12 plants per treatment) was measured at the beginning and end of the photoperiod, from the moment it was visible among the older leaves until the moment it reached the final leaf size, using the soil level as a reference point. Leaf elongation rate (LER) was calculated during steady-state growth phase by dividing the length difference by the time difference between successive measurements. For measurement of meristem size (L_{mer}), the fourth leaf was harvested 2 days after emergence and the basal part of the leaf including meristem was cut into four segments of 1 cm. The leaf segments were fixed in 3:1 (v:v) ethanol:acetic acid solution and stored at 4 °C. Observation was conducted by fluorescence microscopy (DM2500 LED, Leica) at 40× magnification after rinsing the samples in a buffer containing 50 mM NaCl, 5 mM EDTA, and 10 mM Tris-HCl, pH 7, and staining nuclei with 1 mg/ml 4',6-diamidino-2-phenylindole (DAPI) for 1 min. The size of the meristematic zone was defined as the distance from the leaf base to the most distal mitotic cell. For determining the cell length (CL) profile, the growth zone of the fourth leaf, 2 days after appearance, was cut into ten segments of 1 cm and stored in lactic acid following the chlorophyll removal by ethanol. The length of the cells was measured using differential interference contrast microscopy (DM2500 LED, Leica). In each segment, the abaxial epidermal cells located adjacent to stomatal rows in cell files were measured. The raw cell length data obtained for individual leaves were smoothed and interpolated at an interval of 50 μm using Excel 365 statistical tools (Microsoft).

After estimating LER, meristem size and cell length profiles, growth parameters such as cell production (P), division rate (D), cell cycle duration (T_c), number of cells in the meristem (N_{mer}), elongation zone (N_{el}) and mature zone (N_{ma}), cell elongation rate (R_{el}), and time in the elongation zone (T_{el}) were calculated through application of a kinematic analysis approach consisting of formulas described previously (Fiorani et al. 2000).

Flow cytometry

The basal 10 cm of the fourth leaf was cut into segments of 1 cm, except for the first 2 cm which was subdivided further into 5-mm segments. The samples were fixed in liquid

nitrogen and stored at −80 °C until the analysis. The samples were homogenized in 2 ml ice-cold Galbraith buffer [45 mM MgCl₂; 30 mM sodium citrate; 20 mM MOPS; 0.1% (w/v) Triton X-100; pH 7.0] by chopping with a razor blade to release the nuclei. The homogenate was resuspended with a 30-μm nylon filter, and the nuclei were stained with propidium iodide (PI) (1 mg/ml) and analyzed by Accuri C6 flow cytometer (Galbraith et al. 1983).

Transcriptional analysis of cell cycle genes

To analyze cell cycle gene expression, the fourth leaves of seedlings were harvested 2 days after emergence, and the growth zones of the leaves, which were approximately 10 cm from leaf base to tip, were sampled as follows: the first 1 cm for the meristem zone, the 4th cm for the elongation zone, and the 10th cm for the mature zone. Total RNA was extracted using TRI reagent (Sigma-Aldrich). Cell cycle gene identifiers were obtained from Rymen et al. (2007) and the sequence of these genes was retrieved from MaizeGDB (Release 21). Primers were designed with OligoAnalyzer web tool (<https://eu.idtdna.com>) (Table S1). qRT-PCR reactions were performed in triplicate biological and duplicated technical replicates with Applied Biosystems StepOne Real-Time PCR with Power SYBR Green PCR Master Mix (Thermo Fisher). For relative quantification, a threshold cycle was set at the same level for each reaction within the exponential amplification phase. For normalization, the *EF1a* gene was used as a housekeeping gene. Relative gene expression levels were calculated by the $2^{(-\Delta\Delta C_t)}$ method (Pfaffl 2001).

RNA extraction, labeling and microarray hybridization, washing, and staining

Total RNA was extracted from 1 cm (~20 mg) leaf tissue from each growth zone (meristem, elongation, and mature zones) of the fourth leaf using TRI reagent (Sigma-Aldrich). The purified RNA was analyzed by bio-specnano (Shimadzu) and shown to have a 260 nm/280 nm optical density ratio of 1.8–2.0. The RNA integrity was checked by 1% agarose gel electrophoresis. RNA labeling and microarray hybridization, washing, and staining procedures were performed according to the Affymetrix GeneChip miRNA Arrays manual. Three biological replicates were prepared separately for each zone. 10 μg total RNA from each sample was labeled using the FlashTag Biotin HSR kit (Affymetrix) following the manufacturer's protocol. Briefly, this procedure includes poly (A) tailing and FlashTag Biotin HSR Ligation. The labeled samples were hybridized to Affymetrix GeneChip miRNA 4.0 Microarrays at 48 °C and 60 rpm for 16–18 h in an oven (Affymetrix Hybridization Oven 645). The hybridized samples were washed and stained

in an Affymetrix Fluidics Station 450 (FS450_002) following the manufacturer's manual.

Microarray analysis

Microarrays were scanned using Affymetrix GeneChip Command Console (AGCC) and analyzed using Expression Console and Transcription Console software (Affymetrix). Background signal correction and data normalization were applied according to data from spike-in control probes. Statistical analysis was conducted within TAC using one-way ANOVA. Filters criteria applied to data were: ANOVA P value < 0.05 , FDR P value < 0.05 , and logarithm 2-Fold Change $[\log_2FC] > +1$ for upregulated genes and logarithm 2-Fold Change $[\log_2FC] < -1$ for downregulated genes. Only genes that passed the filter criteria were identified as differentially expressed, and these were clustered by hierarchical clustering analysis tools within TAC. Euclidean distances between clusters of objects were computed using the complete linkage method.

In silico prediction of miRNA-target genes

miRNA-target gene candidates were determined using psRNATarget (A Plant Small RNA Target Analysis Server, 2014) web server (Dai and Zhao 2011). Parameters were: maximum expected value = 3, hairpin size = 20; target accessibility = 25, flanking length = 17 bp upstream and 13 bp downstream, and central mismatch = 9–11 nt (<https://plantgrn.noble.org/psRNATarget/>).

Validation of regulation between miRNA and its target mRNAs by qRT-PCR

The differentially expressed miRNAs between growth zones, especially targeting growth-regulatory transcription factors, were selected for miRNA-target validation analysis by qRT-PCR (Livak and Schmittgen 2001). Target mRNA sequences were obtained from MaizeGDB and qRT-PCR primers were designed with OligoAnalyzer web tool as described above. Following Chen et al. (2005), the miRNA stem-loop RT primers were designed as a universal stem-loop structure, extended with six nucleotides that were the reverse complement of the six nucleotides at the 3' end of the mature-miRNA (ma-miRNA) (Table S2). The reverse-transcribed product was amplified using a miRNA-specific forward primer and universal reverse primer (Varkonyi-Gasic et al. 2007).

Total RNA was isolated using TRI reagent (Sigma-Aldrich). miRNA cDNAs were generated with the stem-loop RT primers using 2 μ g total RNA as the template and the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). The stem-loop RT reaction, started by reverse

transcription, subsequently continued with pulsed RT-PCR. The cDNA of the target genes was synthesized using 1 μ g of total RNA with the same kit and random hexamer primers included with the kit. Relative gene expression levels were determined as described above in transcriptional analysis of cell cycle genes. Target mRNA and miRNA levels were normalized with *EF1a* and miR169, respectively, considering that miR169 expression was stable between different developmental stages.

Statistical analysis

Regarding all analyses, Student's t test (two-tailed) was performed using Excel 365 statistical tools (Microsoft). Data were presented as means of three biological replicates \pm standard deviation (SD). Chilling effects were considered statistically significant at $P < 0.05$.

Raw microarray data have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) and can be accessed through the Gene Expression Omnibus series accession number GSE127219.

Results

Effects of chilling stress on maize leaf growth

The effect of chilling stress on the growth of the fourth leaf of maize hybrid ADA313 was investigated through comparative observation among the plants grown under control conditions and the plants subject to low night temperature (4 °C). These conditions did not cause any damage such as discoloring or premature senescence that was confirmed by measurement of photosynthetic pigment content and found that it was not significantly affected (Table 1). However, this low night temperature treatment caused 26% shortening and 35% reduction in the area of the fourth leaf ($P < 0.01$, $n = 12$, Fig. 1a, b). In addition, LER declined about 22% in the fourth leaf of the chilled seedlings during the photoperiod ($P < 0.01$, $n = 12$, Fig. 1c). To analyze the effects of chilling

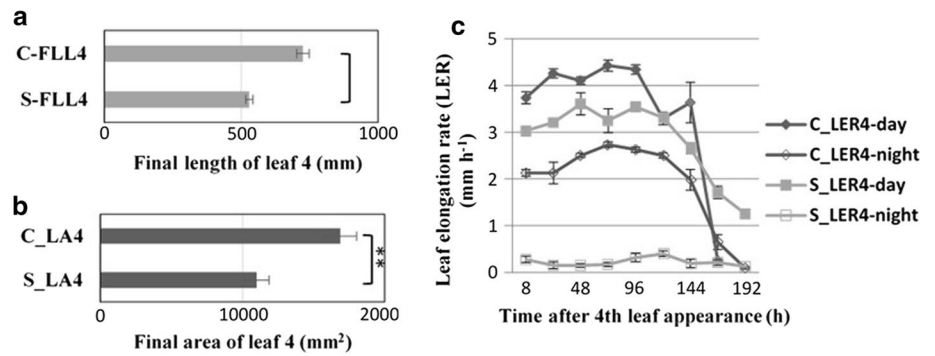
Table 1 Chlorophyll content of the fourth leaf of maize hybrid ADA313 under chilling stress treatment and control condition

	Control	Chilling stress	Difference (%)
Chlorophyll a	26 \pm 2	29 \pm 4	(–)10 (NS)
Chlorophyll b	8 \pm 1	7 \pm 2	(+)2 (NS)
Carotenoid	9 \pm 0	9 \pm 1	– (NS)

NS non-significant, (–) reduction, (+), increase

$n = 3$; average \pm SD; $P < 0.01$, statistical significance based on Student's t test

Fig. 1 **a** Final leaf length (FLL4), **b** leaf area (LA4), and **c** leaf elongation rate (LER) of the fourth leaf of maize hybrid ADA313. LER calculated during night. *C* control, *S* stress. $n = 12$; average \pm SD. $**P < 0.01$, statistical significance based on Student's *t* test



stress at the cellular level, kinematic analysis of the fourth leaf growth was performed during the photoperiod at 48 h after leaf emergence as described above. Epidermal cells were $15.32 \pm 0.91 \mu\text{m}$ and $14.20 \pm 0.37 \mu\text{m}$ in the meristem and expanded to $128.67 \pm 8.98 \mu\text{m}$ and $142.54 \pm 16.31 \mu\text{m}$ in the mature zone of the control and the chilling-treated leaves, respectively (Table 2). Although it was computed that meristematic cell size declined by 7% and increased in the mature zone by 11% in chilling-treated leaves, the differences were not statistically significant. However, *P* decreased significantly by 29% in chilling-treated leaves ($P < 0.05$, $n = 3$, Table 2). Cell production *P* depends on the number of cells in the meristem and on their division rate. Therefore, meristem size L_{mer} was also measured by nuclei staining as the distance from the leaf base to the last mitotic cell. It was found that L_{mer} declined in chilling-treated leaves compared to control cases, but the difference was not significant.

Cell division rate (*D*) was only reduced by 24% (Table 2). The length of the elongation and total growth zone, and the number of cells in those zones remained unchanged under treatment. Therefore, the kinematic analysis has shown that the reduction of growth caused by chilling stress was due to altered cell flux from the meristem to the elongation zone. The reason was slower cell cycle progression; as it was computed, T_c was 32% longer in chilling-treated leaves ($P < 0.05$, $n = 3$, Table 2).

To understand whether the prolonged cell cycle caused by chilling stress occurred during a specific cell cycle phase, the nuclear DNA content was counted by flow cytometry as explained above. According to the profile of nuclear DNA content, the proportion of diploid cells (2C) was the highest in the basal 2 cm, and after that, the proportion of 4C cells began to increase (C is the haploid nuclear DNA content). In the meristem,

Table 2 Effect of chilling stress on cell division and cell expansion parameters which were determined by kinematic analysis on the fourth leaf of maize hybrid ADA313 during steady-state growth

Growth parameters ^a	Control	Chilling stress	% change
Final leaf length (LL) (mm)	718.83 \pm 25.64	532.67 \pm 9.07	(-26)**
Leaf elongation rate (LER) (mm/h)	3.20 \pm 0.12	2.51 \pm 0.02	(-22)**
Mature cell length (CL _{ma}) (μm)	128.67 \pm 8.98	142.54 \pm 16.31	(11)
Meristematic cell length (CL _{me}) (μm)	15.32 \pm 0.91	14.20 \pm 0.37	(-7)
Length of the cell at the end of meristem (μm)	17.22 \pm 0.83	19.03 \pm 2.51	(10)
Cell production (<i>P</i>) (cells/h)	25.02 \pm 1.85	17.86 \pm 1.96	(-29)**
Division rate (<i>D</i>) (cells/cell/h)	0.0259 \pm 0.0015	0.0198 \pm 0.0022	(-24)*
Cell cycle duration (T_c) (h)	26.82 \pm 1.66	35.51 \pm 4.17	(32)*
Length of the meristem (L_{mer}) (mm)	14.80 \pm 1.40	12.8 \pm 0.5	(-14)
Length of the growth zone (L_{gr}) (μm)	77.68 \pm 0.68	83.9 \pm 11.1	(8)
Length of the elongation zone (L_{el}) (mm)	62.91 \pm 0.80	71.07 \pm 11.3	(13)
Number of cells in the meristem (N_{mer})	968.3 \pm 98.67	904.23 \pm 48.32	(-7)
Number of cells in the growth zone (N_{gz})	2107.73 \pm 57.98	2045.59 \pm 144.26	(-3)
Number of cells in the elongation (N_{el})	1139.2 \pm 60.97	1141.36 \pm 107.69	0
Cell elongation duration (T_{el}) (h)	45.98 \pm 6.09	65.18 \pm 12.55	(42)
Cell elongation rate (R_{el})	0.04 \pm 0.00	0.03 \pm 0.01	(-28)

$n = 3$, except for final leaf length and LER, $n = 12$; average \pm SD

^aAll parameters were determined at 48 h after leaf emergence

* $P < 0.05$

** $P < 0.01$, statistical significance based on Student's *t* test

the 2C/4C ratio did not significantly change: the change was only 2.9 ± 0.2 and 2.8 ± 0.1 in control and chilling-treated leaves, respectively. However, in the elongation zone (3–5 cm), the 2C/4C ratio significantly changed by 2.1 ± 0.2 in control leaves and 1.3 ± 0.3 in chilling-treated leaves, respectively ($P < 0.05$, $n = 5$, Fig. 2). This finding indicated that the prolonged cell cycle in chilling-treated leaves was associated with an arrest of the cells in G2 phase. Cells with 8C were also observed in the analysis, suggesting that some 6% of cells proceed to an endoreduplication event. However, this figure was irrespective of chilling treatment.

To confirm whether prolonged cell cycle occurred during a specific cell cycle phase, the expression analysis of core cell cycle genes that are responsible for progression of the cell cycle at the G1/S and G2/M check points was performed following the methodology explained above. To this end, *CYCA2* (S specific), *CYCB2;1* (G2 specific), *CYCD1* (G1 specific), and *CYCD5;1* (G1 specific) genes were selected as marker genes for cell cycle progression.

Results showed that all four *cyclin* genes were highly expressed in meristem, then downregulated in elongation and mature zones ($P < 0.05$, Fig. 3). Although the expression of the G1-specific *CYCD5;1* was the highest in meristem, it was also expressed in elongation and mature zones. However, the expression levels of S- and G2-specific *CYCA2* and *CYCB2;1* were similar in all zones irrespective of the treatment. On the contrary, the expression levels of G1-specific *CYCD1* and *CYCD5;1* were elevated significantly in meristem of chilled treated leaves ($P < 0.05$, Fig. 3). This finding indicated that under the chilling stress condition, cells in the meristem proceeded from G1 to S phase, but the reason for prolonging the cell cycle might be the delay at the G2/M checkpoint.

Fig. 2 Effect of chilling stress on nuclear DNA content along the growth zone of leaf 4 (10 cm) which was determined by flow cytometry. Representative fluorescence intensity distributions of samples from meristem (a), elongation (b), and mature (c) zones under control conditions. **d** DNA amount from leaf base to tip under chilling and control conditions. G1 and G2, cell cycle phases; 2C, 4C, and 8C represent ploidy level. C control, S stress. $n = 5$; average \pm SD. * $P < 0.05$, statistical significance based on Student's *t* test

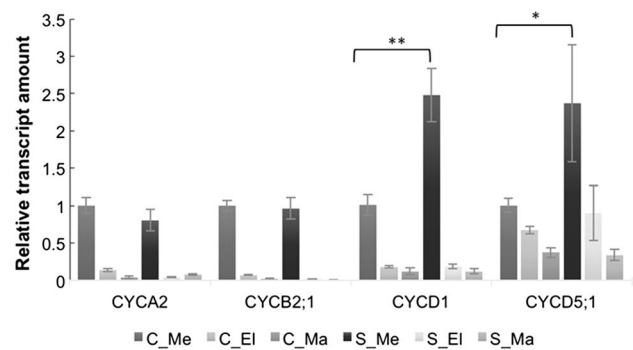
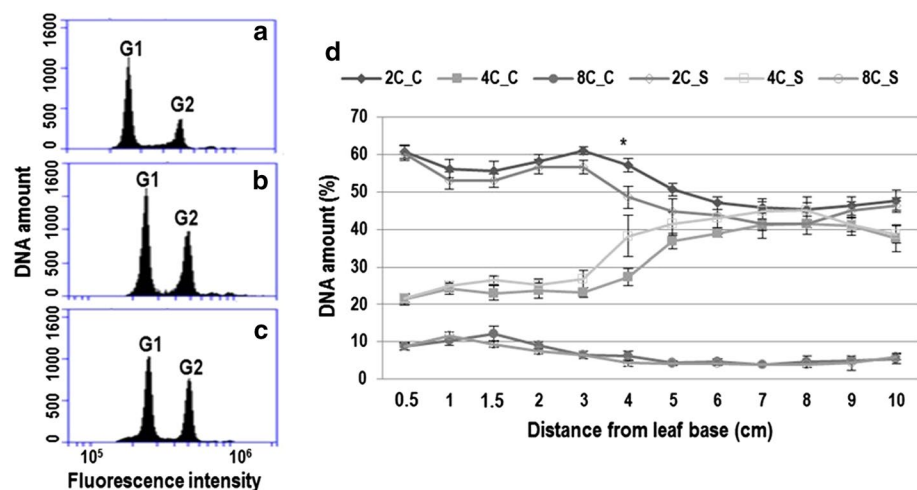


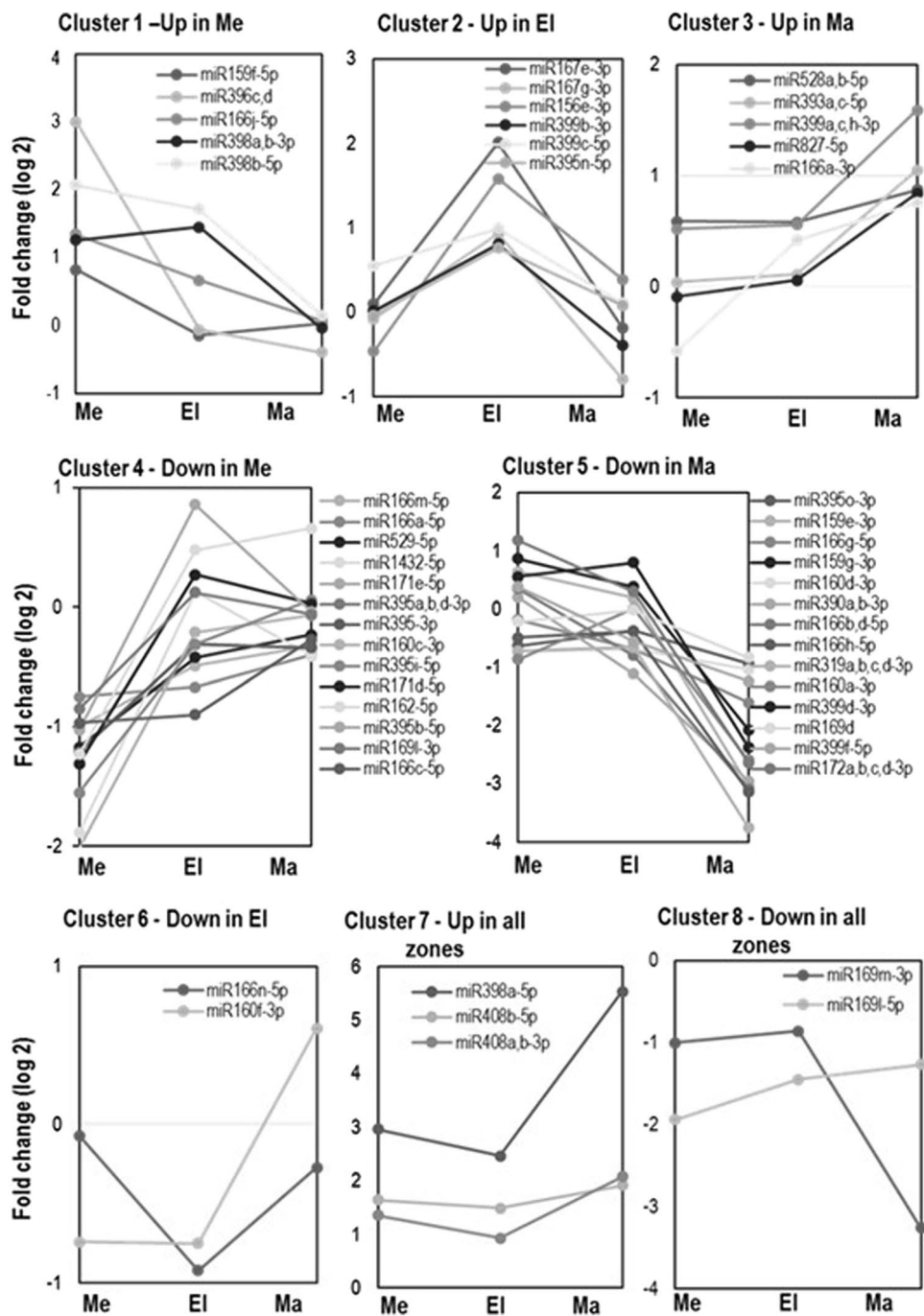
Fig. 3 The expression profiles of cell cycle check point genes in the meristem (Me, 0–1 cm), elongation (El, 3–4 cm), and mature (Ma, 9–10 cm) zones were determined using qRT-PCR. Results were presented as relative to control meristem. $n = 3$; average \pm SD. * $P < 0.05$, ** $P < 0.01$, statistical significance based on Student's *t* test

Expression profiles of putative chilling-responsive growth-regulatory miRNAs of maize (*Zea mays* L.) hybrid ADA313

To identify miRNAs having regulatory roles in leaf growth under chilling stress, the expression profiles of 321 known maize miRNAs were determined using an miRNA microarray (miRNome) analysis as described above. Out of 321 known maize, miRNAs with a log fold change of $-1 < \text{log fold change} < +1$ were divided into eight clusters according to their expression profiles across the growth zones and their response to chilling stress in each zone (ANOVA P value < 0.05 , FDR P value < 0.05 , Fig. 4).

The miRNAs in the first six clusters were assigned to meristem, elongation, or mature zones (Fig. 4). 24 miRNAs were specifically up or downregulated in meristem, 6 miRNAs in elongation zone, and 20 miRNAs were specific to mature zone (Fig. 5). It was found that 14 miRNAs were upregulated and 23 miRNAs were downregulated in

Fig. 4 miRNA genes clusters that are significantly differentially expressed between the maize leaf growth zones under chilling stress. Clusters were produced by Affymetrix Transcriptome Analysis Console. Me, El, and Ma represent meristem, elongation, and mature zones, respectively. Cluster 1, -2, -3: miRNAs were upregulated in meristem, elongation, and mature zones, respectively; Cluster 4, -5, -6: miRNAs were downregulated in meristem, mature, and elongation zones, respectively; Cluster 7, miRNAs were upregulated in all zones; Cluster 8, miRNAs were downregulated in all zones. $n=3$; $P<0.05$, one-way ANOVA with FDR correction; $\log_2FC > +1$ and $\log_2 < -1$



meristem, 12 miRNA were upregulated and 5 miRNAs were downregulated in elongation, and 9 miRNAs were upregulated and 23 miRNAs were downregulated in the mature zones (Fig. 5).

Putative targets of chilling-responsive growth-regulatory miRNAs

Because miRNAs do not directly modulate plant responses to stress, but rather these are involved in the degradation

of target genes, identification of the target genes that are participated in chilling stress responses is essential to reveal the regulatory roles of the miRNAs in stress tolerance. From the kinematic analysis, it was shown that the growth inhibition caused by chilling stress was mainly due to prolonged cell division. Therefore, miRNAs possibly related to meristematic functions and their putative targets were highlighted. In silico prediction tools, it was shown that putative chilling-responsive growth-regulatory miRNAs targeted many developmental transcription factors such as *MYB transcription*

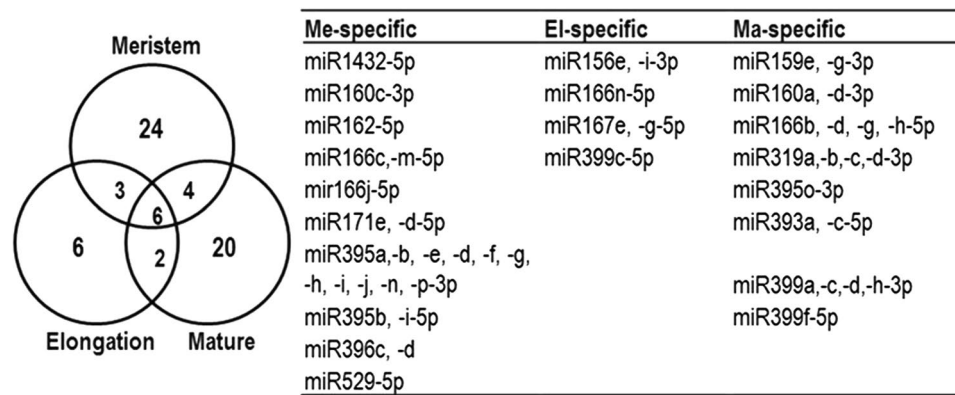


Fig. 5 Overview of the miRNAs changed significantly in response to chilling stress in each developmental zone [meristem (Me), elongation (El), and mature (Ma)] along the leaf axis. Numbers represent individual miRNAs. Letters represent the family members within a

miRNA family. $n=3$; $P<0.05$, one-way ANOVAs with FDR correction; $\log_2FC>1$, for upregulated genes; $\log_2FC<-1$, for downregulated genes

factors (*MYBs*), *Gibberellin- and Abscisic acid-regulated MYB (GAMYB)*, *TEOSINTE BRANCHED1* in *Zea mays*, *CYCLOIDEA* in *Anthirrinum majus*, and *PCF (Proliferating Cell nuclear antigen Factor)* in *Oryza sativa* (*TCPs*), *Growth Regulating Factors (GRFs)*, and *Auxin Responsive Factor transcription factors (ARFs)* (Table S3).

Validation of regulation between putative chilling-responsive growth-regulatory miRNAs and their predicted targets

To validate possible interactions between putative chilling-responsive growth-regulatory miRNAs and their in silico predicted targets, expression profiles of the selected miRNAs and their putative target transcription factors were analyzed by qRT-PCR. To this end, target genes of the selected miRNAs were specified as: *MYB87*, *GAMYB*, *TCP5*, and *TCP38* for miR319; *ARF15*, *ARF17* for miR160d-5p; *GRF1*, *GRF5*, *GRF15* for miR396; *Wall-associated kinase 3 (WAK3)* for miR1432-5p; *3-oxo-Delta(45)-steroid 5-beta-reductase (VEP1)* for miR408a; *superoxide dismutase (SOD)* for miR528, and *tRNA (cytosine(34)-C(5))-methyltransferase-like isoform X2 (CMT2)* for miR395i-5p. Five candidates of the selected miRNAs exhibited good correlations between microarray and qRT-PCR, and the results were demonstrated by the scatter plots in Fig. S1.

miR319 was expressed highly in the meristem zone and downregulated in elongation and mature zones ($P<0.05$, Fig. 6a). In response to the chilling stress, it was found that the expression of miR319 in the mature zone decreased significantly compared to the control group ($P<0.05$). When the expression of the predicted targets was examined, it was found that the *MYB87* had a high expression in the meristem and decreased expression in the mature zones similar to miR319 ($P<0.05$). The expression of *TCP5* was also

high in the meristem and decreased in the elongation and mature zones; however, the expression in the meristem in response to chilling decreased significantly compared to the control group ($P<0.05$). By contrast, other predicted targets of miR319, *GAMYB*, and *TCP38* were lowly expressed in the meristem and highly expressed in elongation and mature zones. Under chilling stress, *TCP38* was significantly downregulated in elongation and mature zones compared to the control group, whereas *GAMYB* was significantly downregulated only in the mature zone ($P<0.05$).

miR160 expression significantly decreased in mature zone compared to meristem and elongation zones ($P<0.05$) and remained unchanged in response to chilling stress ($P>0.05$) (Fig. 6b). *ARF15* was found highly expressed in the meristem and elongation zones and lowly expressed in mature zone. During the stress response, *ARF15* was downregulated in the elongation and upregulated in the mature zones. The expression of *ARF17*, other predicted target of miR160, did not change in the meristem and elongation zones; however, it was upregulated in the mature zone compared to the meristem and elongation zones and downregulated in response to chilling stress ($P<0.05$).

A low miR396 expression was observed in the meristem and a high expression in elongation and mature zones ($P<0.05$) (Fig. 6c). All three predicted targets, *GRF1*, *-5*, and *-15*, showed a reciprocal expression pattern that are high in meristem but low in elongation and mature zones ($P<0.05$). In response to low temperature, it was detected that *GRF5* decreased in meristem zone, *GRF1* increased in meristem and decreased in mature zones, and *GRF15* decreased in mature zone with no significant variation was detected for miR396 ($P<0.05$).

miR408a expression was significantly higher in elongation and mature zones compared to meristem zone under control conditions ($P<0.05$) (Fig. 6d). However, in

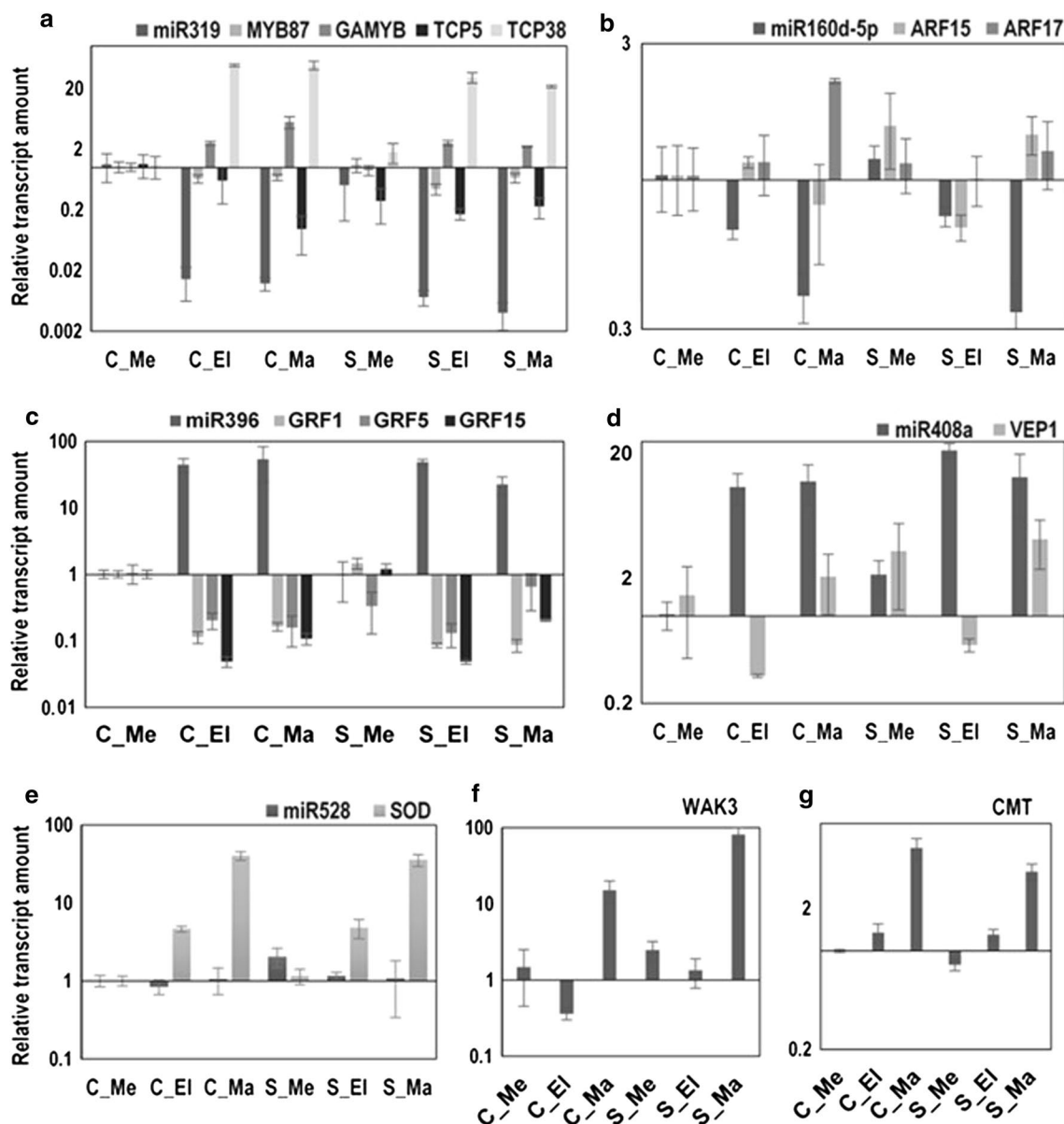


Fig. 6 Validation of relationship between putative chilling-responsive growth-regulatory miRNAs and their target genes by expression analysis. **a** miR319 and its targets. **b** miR160 and its targets. **c** miR396 and its targets. **d** miR408 and its targets. **e** miR528 and its targets. **f** Target of miR1432. **g** Target of miR395. C_Me, Control meristem; C_El, Control elongation; C_Ma, Control mature; S_Me, Stress meristem; S_El, Stress elongation; S_Ma, Stress mature. MYB74 (*MYB transcription factor 74*); TCP5, -38 (*TEOSINTE BRANCHED1*,

CYCLOIDEA, and *PCF* coding gene like transcription factors 5,-38), *GAMYB* (*Gibberellin- and Abscisic Acid-regulated MYB*); *ARF15* (*Auxin responsive factor transcription factor 15, -17*); *GRF5* (*Growth Regulating Factor 1, -5, -15*); *VEP1* (*3-oxo-Delta(45)-steroid 5-beta-reductase*); *SOD* (*superoxide dismutase*); *WAK3* (*Wall-associated kinase 3*); *CMT2* (*tRNA (cytosine(34)-C(5))-methyltransferase-like isoform X2*). $n=3$; average \pm SD; $P < 0.05$, statistical significance based on Student's *t* test

response to chilling stress, expression in the meristem and elongation zones increased, while it stayed unchanged at mature zone ($P < 0.05$). *VEP1* was predicted as target for miR408, and its expression decreased in the elongation zone compared to meristem and mature zones. In addition, it was significantly upregulated by twofold in those growth zones under the effect of chilling stress ($P < 0.05$).

The expression of miR528 did not differ between the growth zones under control conditions, but increased in the meristem in response to chilling stress (Fig. 6e). The expression of its predicted target *SOD* showed an increasing profile from the meristem to the mature zone. The expression of miR1432-5p and miR395i-5p was not detected by stem-loop RT-PCR. However, their predicted target genes, *WAK3* and *CMT2*, were

found to be upregulated and downregulated, respectively in the mature zone in response to chilling ($P < 0.05$) (Fig. 6f, g).

Discussion

Chilling causes dramatic growth inhibition on maize hybrid ADA313

This study was designed to investigate the role of microRNA (miRNA) genes in rearranging plant growth functions during the chilling stress response regarding maize hybrid ADA313. To this end, the fourth leaf of maize seedlings was used as a model and its growth response to stress treatment was monitored from emergence to maturity. Stress duration and severity were designed to avoid damage to the photosynthetic apparatus aiming at monitoring direct interactions between growth and miRNA regulation under chilling stress. However, leaf length (LL) significantly declined by 26% in response to low night temperatures, which was accompanied by 22% decrease in LER ($P < 0.01$).

To identify the reason for the 26% reduction in leaf length (LL) at the cellular level, a kinematic analysis was conducted to determine the growth parameters such as P , cell division rate (D), T_c , and cell elongation rate (R_{cl}) (Fiorani et al. 2000; Schnyder et al. 2009). Jones et al. (2017) showed that proceeding through G1/S and G2/M cell cycle check points is related to cell size rather than cell age. Accordingly, large cells divide more quickly. However, in microscopic measurements, no significant difference was found between the cell length profiles of seedlings grown in control conditions and chilling-treated ones (Table 2). Since the final size of an organ depends on both the number and size of its cells, and the cell expansion functions were not significantly affected, the 26% change in LL could be due to cell production (P). Accordingly, the kinematic analysis showed that P declined significantly in chilling-treated leaves (29%, $P < 0.05$, Table 2). Since P depends on cell number and division rate in the meristem, meristem size L_{mer} was determined by nuclear staining. There was a small but not statistically significant difference in L_{mer} . On the other hand, chilling stress caused reduction in cell division rate D (24%, $P < 0.05$, Table 2). In the light of these findings, it was concluded that the shortened leaves in chilling-treated seedlings were related to reduced P resulting from slowed cell cycle progression. In fact, it was indicated that the chilling stress caused a 32% increase in T_c ($P < 0.05$, Table 2).

Chilling stress inhibits leaf growth by disturbing cell cycle progression

Cell cycle progression in chilling stress-treated and control seedlings was observed in more detail by measuring DNA content along the growth zones by flow cytometry and

relative expression of cyclin genes functioning at the cell cycle check points by qRT-PCR. Flow cytometry analysis has supported the finding that leaf growth inhibition was tightly associated with the reduction of P as a consequence of prolonged T_c , but did not reduce cell number or cell size in the meristem.

The DNA content of cells in the meristem and mature zones of control leaves were found to be less compared to those of chilling-treated leaves, but the difference was not statistically significant ($P > 0.05$, Fig. 2). However, in the elongation zone, the 2C/4C ratio significantly declined in chilled leaves, indicating that the increased duration of the cell cycle calculated by the kinematic analysis in the leaves exposed to chilling was a result of arrest in G2 phase. Therefore, while the cells doubled their amount of DNA, the prolongation of the G2 phase led the cells leaving the meristem zone without dividing. These findings have confirmed previous studies focusing on maize as these indicated that G2 phase cells are more sensitive to extreme temperatures (Rymen et al. 2007). By contrast to this study, it was shown that osmotic stress acts specifically on the G1/S transition or S/G2 transition (Zhao et al. 2014).

Previous studies demonstrated that plant growth response to various stresses including cell cycle modifications mediated by *cyclin-dependent kinases* (CDKs) and the *cyclin* genes involved in G1/S and G2/M cell cycle control points (Menges et al. 2005; Zhao et al. 2014). Based on these studies, *CYCA2* (S specific), *CYCB2;1* (G2 specific), and *CYCD1* and *CYCD5;1* (G1 specific) were selected as marker genes to give information about cell cycle progression. As expected, qRT-PCR analysis showed that all these *cyclins* were upregulated in the meristem where active cell production takes place and downregulated in the elongation and mature zones. However, it was also found that G1-specific *cyclin* genes, especially *CYCD5;1* continued to be expressed at decreasing levels in the elongation and mature zones (Fig. 3). There was no statistically significant difference in the expression of the G2/M-specific *CYCA2* and *CYCB2;1* due to stress ($P > 0.05$). However, the expression of the G1/S-specific *CYCD1* and *CYCD5;1* increased significantly in the meristem in response to chilling stress ($P < 0.05$). High expression of *D-type cyclin* is expected to cause cells to advance through the G1/S checkpoint; however, the relatively low expression of *A- and B-type cyclins* suggests that cells would remain longer in S phase and G2 phase leading to higher proportion of 4C cells observed in the elongation zone. Similarly, Zhao et al. (2014) examined the expression of *cyclin* genes under various stresses in maize and they observed that *CYCD5;1* significantly increased as exposed to cold treatment. In addition, they showed that cyclin-dependent kinase *CDKA* is constitutively expressed throughout the cell cycle, but its expression decreased in response to abiotic stresses, which indicated that cell cycle progression was blocked. While

mannitol treatment caused downregulation of *D-type cyclin* accompanied with cell cycle arrest in G1 phase, heat and cold treatments, both led a retention in G2/M with decreased *B-type cyclin* expression. On the other hand, CuSO₄ treatment led to downregulation of *A-type cyclins*, resulting in blocking the cells in S phase. Jones et al. (2017) showed that cell size is directly related to the developmental stage of the plant and environmental factors that regulate the duration of the cell cycle. All these findings suggest that chilling stress does not lead to a defect in the G1 phase of the cell cycle, but should be arrested at the G2/M stage.

Chilling-responsive miRNAs differentially expressed between leaf growth zones and regulate developmental transcription factors

Genome-wide expression profiling of 321 known maize miRNAs through application of miRNome analysis has identified eight clusters according to differential expression profiles of miRNAs in the corresponding growth zones under chilling stress (Fig. 4). Furthermore, 24, 6, and 20 miRNAs were assigned to meristem, elongation, and mature zones, respectively, being specific chilling-responsive miRNAs (Fig. 5).

Following the miRNome analysis, in silico target prediction was conducted for the putative meristem-specific miRNAs, considering that kinematic analysis pointed out this zone through revealing the decreased cell production. Each miRNA was predicted to target more than one gene. As low-temperature molecular responses are frequently mediated by signalling pathways such as ABA, SA, and CBF/DREB, the predicted targets were searched for members of these pathways (Miura and Furumoto 2013). No gene related to those pathways was directly targeted by an miRNA, but some transcription factors that function together with hormones were predicted as targets. There was one miRNA (miR528) that was also selected as a putative regulator of reactive oxygen species (ROS) scavenging, because maintenance of redox homeostasis is important for continuation of growth and development under stress. Finally, some developmental transcription factor genes were selected. Targets to be validated were as follows; *MYB87*, *GAMYB*, *TCP5* and *TCP38* for miR319; *ARF15*, *-17* for miR160d-5p; *GRF1*, *-5* and *-15* for miR396; *WAK3* for miR1432-5p; *VEP1* for miR408a; *CMT2* for miR395i-5p; and *SOD* for miR528 (Tables S2 and S3).

The expression of miR319 was high in the meristem that is consistent with its indirect role in maintaining the cell division function of the meristem and significantly downregulated in the mature zone in response to chilling stress ($P < 0.05$, Fig. 6a). No inverse correlation was detected between expression of miR319 and predicted targets *MYB87* and *TCP5*, both of which displayed similar expression profiles to miR319. However, other predicted targets such as

TCP38 and *GAMYB* were reciprocally expressed to miR319 which pointed to putative regulation between them. Both miR319 and putative targets were downregulated in mature zone in response to chilling which indicated that they are not specifically involved in the chilling response ($P < 0.05$). *TCPs* are plant-specific growth-regulating transcription factors that trigger cell division with suppressing cell differentiation. Studies on Arabidopsis have shown that high *TCP4* activity in the meristem resulted in decreased cell production (Schommer et al. 2014). miR319 was shown to be a positive regulator of cold tolerance through targeting *PCF* and *TCP* genes in rice (Yang et al. 2013; Wang et al. 2014). *MYBGA* or *GAMYB* encodes a subset of highly conserved *R2R3-MYB* transcription factors that control various developmental processes in response to plant hormones including gibberellin (GA) and ABA (Woodger et al. 2003). Similar to these findings, Nelissen et al. (2018) observed that GA levels increased from the leaf base to tip in GA-overexpressing maize mutants and concluded that GA had an important role in plant development. In summary, these findings in the literature indicate a possible role for miR319 in maintaining cell division in the meristem by suppressing *TCP38* and *GAMYB*. Chilling stress did not significantly alter expression of these genes in the meristem, indicating that they are not directly involved in the stress response. However, both targets and miR319 were downregulated in response to chilling in the mature zone pointing out a possible consequence of a general suppression of transcription.

miR160 was highly expressed in the meristem and downregulated in elongation and mature zones. However, it was slightly upregulated in meristem in response to low temperature causing differential expression patterns between meristem and elongation ($P < 0.05$, Fig. 6b). The expression of predicted target *ARF15* was not correlated with miR160, but was downregulated in the elongation zone and upregulated in the mature zone in stress-treated leaves. The expression of *ARF17*, the other predicted target of miR160, was upregulated in maturity compared to the meristem and elongation zones but downregulated in response to chilling stress ($P < 0.05$). The reciprocal expression profiles of miR160 and *ARF17* suggest that miR160 may regulate this transcription factor. ARFs bind to auxin response elements (AuxREs) playing a key regulatory role in almost every step of plant growth and development (Li et al. 2016). Evidence from Arabidopsis has shown that the expression of *ARFs* is dynamic and the expression profile changes depending on the stage of plant development; and that different *ARFs* control different development processes (Rademacher et al. 2012). Regarding maize, Liu et al. (2011) identified 31 maize *ARF* genes which are expressed in various tissues or organs. Specifically, they found that *ARF17* and *-25* were expressed in maize leaf. In addition to their findings, the current study showed that *ARF15* was also expressed in maize leaf.

The expression profile of miR396 and its predicted targets including *GRF1*, *-5*, and *-15* were also reciprocal to each other. *GRFs* were highly expressed in meristem in the absence of miR396 to promote growth functions, but this was reversed in the elongation and mature zones. In response to low temperature, the expression of *GRF5* decreased in the meristem, *GRF1* increased in the meristem and decreased in maturity zone, and *GRF15* decreased in maturity zone ($P < 0.05$, Fig. 6c). These fluctuations may result from compensation between *GRFs* under stress conditions. *GRFs* are plant-specific growth regulating transcription factors that form a complex structure with a co-activator *GIF* (*GRF interactive factor*) (Kim and Tsukaya 2015). Studies on miR396-resistant maize mutant where *GRF* was highly expressed have shown that these may cause larger leaves by elevation of the number of dividing cells (Nelissen et al. 2015). In another study focusing on Arabidopsis, through overexpressing miR396-insensitive *GRF3* and *GIF1*, it was found that the cell number of leaves were increasing (Debernardi et al. 2014). Also, Arabidopsis mutants overexpressing miR396a or miR396b had narrowed leaf phenotype due to decreased cell number (Liu et al. 2009). This study was consistent with miR396 regulating its predicted targets *GRF1*, *-5*, and *-15* but no specific correlation was detected with the chilling stress response.

The expression of miR408 was significantly higher in the elongation and mature zones than in meristem under control conditions; it was further upregulated in the meristem and elongation zones under chilling stress ($P < 0.05$, Fig. 6d). However, the expression profile of it and its predicted target *VEP1* were not reciprocal, suggesting that it may not be the primary regulator of *VEP1* ($P < 0.05$). However, it may have a regulatory role in regulation of other genes in the meristem and elongation zones during chilling stress. This was supported by Ma et al. (2015) who found that miR408 in Arabidopsis conferred tolerance during salinity, cold, and oxidative stress, but had increased sensitivity to drought and osmotic stress. Another maize microarray study by Wu et al. (2014) found that miR408 was one of four biotic stress-responsive miRNAs. A more recent study by Bai et al. (2018) uncovered the role of miR408 in responding to inorganic phosphate deficiency in wheat plant. All these findings indicate that manipulation of miR408 may be a useful agricultural practice in increasing stress tolerance in maize plants. However, the reciprocal expression between miR408 and *VEP1* was observed only in the elongation zone. Therefore, new prediction tools should be employed for identifying other targets to determine possible mechanisms for this regulation.

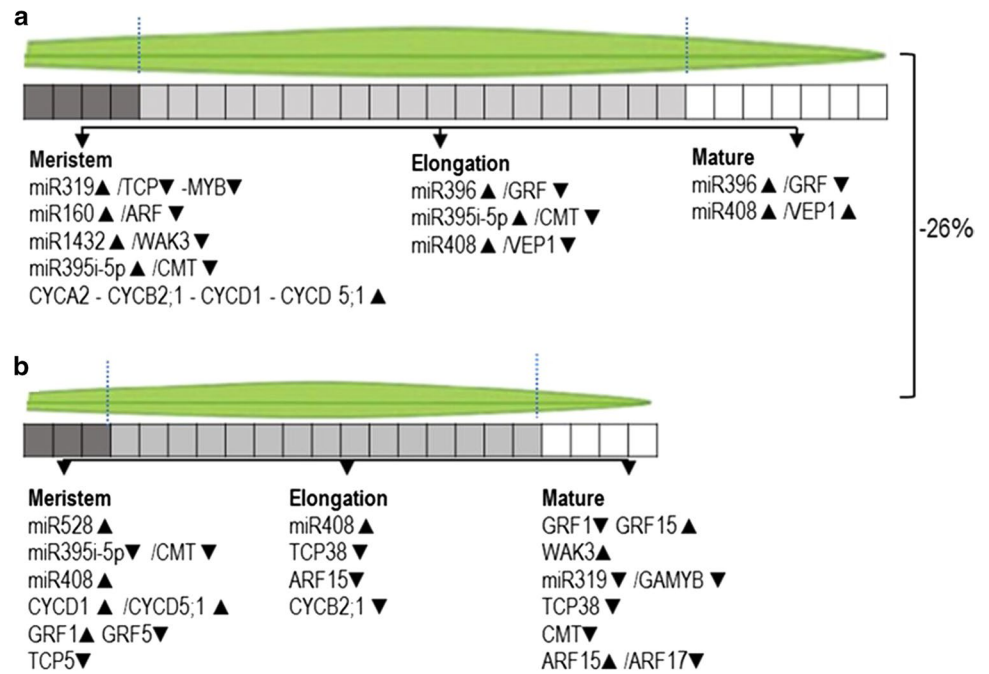
The expression of miR528 did not differ between the growth zones under control conditions but increased in the meristem in response to chilling stress ($P > 0.05$, Fig. 6e). The expression of its predicted target *SOD* showed an increasing profile from meristem to the mature zone. These results indicate that miR528 was meristem-specific and played a role in response to chilling stress. Further to this, *SOD* would also be regulated by another regulation mechanism besides miR528 as being an important enzyme maintaining redox homeostasis under stress conditions. Eventually, the possible interaction between them needs to be confirmed by further analysis.

Two miRNAs, miR1432-5p and miR395i-5p, were detected as meristem-specific and chilling stress responsive from the microarray data. However, they were not detected by stem-loop qRT-PCR, which might be a consequence of mutations in the mature sequence of these miRNAs in ADA313. Despite this, their predicted targets *WAK3* and *CMT2* were found to be expressed in the mature zone in both control and chilling stress conditions. In addition, *WAK3* expression at the mature zone increased as a response to stress, while expression of the *CMT2* gene declined ($P < 0.05$, Fig. 6f, g).

Conclusion

This study has shown that chilling stress retarded leaf growth by inhibiting cell cycle progression through the G2/M checkpoint in hybrid maize ADA313. Furthermore, microarray analysis revealed a large number of miRNA with altered expression in different developmental zones in response to chilling stress, many of which were verified by qRT-PCR (Fig. 7). The reported role of miR319 and miR396 in regulating transcription factors involved in leaf development was confirmed, but this system did not appear to be directly involved in the response to chilling stress. Conversely, miR408 and miR528 were highlighted as chilling-responsive miRNAs but not their targets. Therefore, mechanisms of action still need to be determined among them. For future research, these interactions should be elucidated in more detail to facilitate the manipulation of miRNA pathways by biotechnological methods, aiming at enlarging the leaf size and allowing more effective light capture, particularly under stress conditions.

Fig. 7 Modeling of chilling response of the fourth leaf of maize based on kinematic and expression data. **a** Control leaf and the genes that are differentially expressed between growth zones. **b** Chilled-treated leaf and the genes that are differentially expressed response to treatment in growth zones



Author contribution statement FA designed research, performed experiments, interpreted data and wrote the paper.

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