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Addressing the role of microRNAs in reprogramming leaf growth during drought stress in *Brachypodium distachyon*

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Running Title: miRNAs during drought stress in Brachypodium leaf

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ABSTRACT

Plant responses to drought are regulated by complex genetic and epigenetic networks leading to rapid reprogramming of plant growth. miRNAs have been widely indicated as key players in the regulation of growth and development. The role of miRNAs in drought response was investigated in young leaves of *Brachypodium distachyon*, a drought tolerant monocot model species. Adopting an *in vivo* drought assay, shown to cause a dramatic reduction in leaf size, mostly due to reduced cell expansion, small RNA libraries were produced from proliferating and expanding leaf cells. Next-generation sequencing data were analyzed using an in-house bioinformatics pipeline allowing the identification of 66 annotated miRNA genes and 122 new high confidence predictions greatly expanding the number of known Brachypodium miRNAs. In addition we identified four TAS3 loci and a large number of siRNA producing loci that show characteristics suggesting that they may represent young miRNA genes. Most miRNAs showed a high expression level, consistent with their involvement in early leaf development and cell identity. Proliferating and expanding leaf cells respond differently to drought treatment and differential expression analyses suggest novel evidences for a miRNA regulatory network controlling cell division in both normal and stressed conditions and demonstrate that drought triggers a genetic reprogramming of leaf growth in which miRNAs are deeply involved.

Keywords: miRNAs, drought, Brachypodium, leaf development

Introduction

Current projections on global environmental changes and population growth point to the necessity for a 70% increase in food production by the next mid-century (FAO, 2006; Godfray et al., 2011; Tester and Langridge, 2010). Particularly in the context of climate change, water scarcity is increasing worldwide and drought stress is a critical environmental factor whose effects on crop yields are mediated in part through alterations in plant development, metabolism and gene expression (Ceccarelli and Grando, 1996). For these reasons, understanding abiotic stress responses is a major objective of plant research and a primary motivating factor for future breeding programs (Godfray et al., 2011; Tester and Langridge, 2010).

Much progress has been achieved in this field, producing a complex picture of plant responses to growth limiting environments (Hirayama and Shinozaki, 2010). Modulation of several metabolic pathways under drought stress conditions has been described (Seki et al., 2007), revealing biochemical and physiological mechanisms that help to balance water uptake and loss and protect cells from damage. Additionally, key components of abiotic-stress responsive regulatory networks, mediated by abscisic acid (ABA) dependent and independent signal transduction pathways, have been isolated and characterized (Shinozaki and Yamaguchi-Shinozaki, 2007; Zou et al., 2011). For the most part these mechanisms have been studied in mature tissues and organs (e.g. whole leaf and root) and considered as unique and distinct components that respond uniformly to stimuli. However it is now emerging that stress responses can be specific to developmental stages and cell types (Hausmann et al., 2005; Iyer-Pascuzzi et al., 2011; Skirycz et al., 2011; Skirycz et al., 2010). Indeed, it is now recognized that, on exposure

to stress, plants reduce their growth rates to save energy and redistribute resources, establishing a new growth program involving multiple tissues and developmental stages. Furthermore, such responses should not be interpreted exclusively as measures to compensate for reduction in photosynthetic activity (Fricke et al., 2006; Veselov et al., 2002).

In the past decade, a variety of pathways in which small RNAs (sRNA) guide gene silencing through sequence complementarity-dependent transcriptional and post-transcriptional mechanisms have been described. Plants encode 21–24 nt long small RNAs molecules, which, depending on their mechanism of biogenesis, are classified as microRNAs (miRNAs), trans-acting siRNAs (ta-siRNAs), natural antisense siRNAs (nat-siRNAs) and repeat-associated siRNAs (ra-siRNAs) (Vaucheret, 2006). MicroRNA as are the best studied class of small RNAs and include the most abundantly expressed individual sequences. miRNAs are cleaved by an RNaseIII enzyme (DICER-like) as duplexes, comprising the mature miRNA and its complementary sequence, known as the miRNA*, from long double-stranded RNAs precursors. In plants they are generally transcribed from dedicated microRNA (MIR) genes. After maturation miRNAs associate with a member of the ARGONAUTE family in RNA-induced silencing complexes (RISCs). RISC exerts its effect in RNA silencing by facilitating recognition of RNA sequences showing complementarity to incorporated small RNAs. Target RNAs are then cleaved or rendered unavailable to translation (reviewed in (Chen, 2009)).

To date, various experimental and *in silico* approaches have been used to identify thousands of miRNA genes in plants, animals and viruses. Rules to define plant miRNA producing loci have been strictly defined in order to avoid misleading annotation and

conflation of different classes of small RNAs (Meyers et al., 2008). As a consequence, all siRNAs that resemble miRNAs, but do not conform to these strict criteria, are called siRNA-like miRNAs. Plant miRNAs are known to play pivotal roles in a variety of physiological and developmental processes, such as organ development, phase transition, flowering, genome maintenance and response to environmental stimuli including biotic and abiotic stresses (Chitwood et al., 2009; Ding and Voinnet, 2007; Dunoyer et al., 2010; Lu et al., 2005; Molnar et al., 2010; Rubio-Somoza et al., 2009; Rubio-Somoza and Weigel, 2011; Sunkar, 2010)

Indeed, many aspects of leaf development including abaxial and adaxial polarity, the definition of the medio-lateral and proximo-distal developmental axes, meristem identity and adult phase transitions are all governed by different miRNAs (Kidner, 2010; Pulido and Laufs, 2010). Many of these studies were performed in Arabidopsis or in the monocots *Zea mays* (Lauter et al., 2005; Nogueira et al., 2009; Nogueira et al., 2007) and *Oryza sativa*, where the involvement of miRNAs in abiotic stress response has also been investigated (Shen et al., 2010; Zhao et al., 2007; Zhou et al., 2010).

Of these, miRNA involvement in leaf development along the proximo-distal axis has been less investigated, especially in monocots where cells are arranged in rows determining a developmental gradient of cells along the longitudinal axis. At the leaf basis all cells are proliferating and at a given distance from the leaf basis cells will cease division and start to expand and differentiate. Recently, gibberellins were shown to have a key-role in regulating the transition from cell proliferation to cell expansion (Nelissen et al., 2012).

In *Brachypodium distachyon,* a drought tolerant undomesticated species that has become a model plant for temperate cereals (Brkljacic et al., 2011; Vogel et al., 2010),

it has been recently shown that, in contrast to the situation in Arabidopsis and maize, the meristem is nearly unaffected by drought, and reduced leaf length is entirely caused by a reduction of cell expansion (Verelst et al., 2012) In the present study we have used next generation sequencing (NGS) technology to annotate and profile the expression of conserved and non-conserved miRNA and miRNA-like molecules during Brachypodium leaf development along the proximo-distal axis and compare miRNA expression along this axis during normal and severe drought conditions. Specifically, we have identified a total of 270 miRNA and miRNA-like genes, confirming 66 previous annotations (Baev et al., 2011; Unver and Budak, 2009; Wei et al., 2009; Zhang et al., 2009) and adding 28 additional loci from known miRNA families as well as 94 novel miRNA genes plus 82 siRNA-like miRNA loci. Differential expression analyses suggest that a number of miRNAs are involved in developmental reprogramming of leaf growth in response to drought. This study provides additional evidence for a miRNA regulatory network controlling cell division in both normal and stressed conditions.

Results

High-throughput sequencing of small RNAs from Brachypodium distachyon

As described in Methods, we set up an *in vivo* drought assay of Brachypodium plants, shown to cause a dramatic reduction in leaf size mostly affecting cell expansion (Verelst et al., 2012). We collected the 3rd leaf (leaf 3) of Brachypodium plants grown in control and stress condition (Bd21 inbred line). Leaves were sectioned and small RNA libraries were generated from proliferating (P) and expanding (E) leaf zones of plants grown

under drought stress (s) and control conditions (c). For each condition, two biological replicates were considered for a total of 8 libraries (Ps1, Ps2, Pc1, Pc2, Es1, Es2, Ec1, Ec2). Each library was subjected to deep sequencing using the Illumina GAII platform generating between 19.7 and 30.1 million raw reads. After adapter trimming and removal of low quality reads, 16 - 22.7 million reads between 18 nt and 26 nt in length per library were subjected to further analyses (see Supplementary Table 7 online for details for each library). Reads were mapped to the *B. distachyon* Bd21 genome sequence (Vogel et al., 2010) using SOAP (Li et al., 2008a), retaining only sequences with a perfect match to the reference sequence (on average 92% of trimmed reads). Reads derived from known non-coding structural RNAs (tRNAs and rRNAs) were excluded from subsequent analyses (Supplementary Table 7 online).

The distribution of sizes of sequenced small RNAs (Figure 1) is similar for all samples and, as expected, shows peaks at 21 and 24 nt. Twenty-four nt long molecules, consisting mainly of siRNAs, are the most abundant in all the samples, but on average each unique sequence has a low redundancy. Conversely, we observe fewer distinct 21nt molecules but on average, each unique 21mer shows higher representation in the libraries (see Figure 1 and Supplementary Figure 1 online) consistent with the expected pattern for miRNAs.

Identification of conserved and non conserved microRNA families

An *ad-hoc* bioinformatics pipeline was used to annotate known and unknown miRNAs, both conserved and lineage-specific. In the present manuscript, all miRNAs belonging to families already annotated in the miRBase Registry (www.mirbase.org, Release 18) in at least one species are defined as *known* (Axtell and Bowman, 2008), while we

define as *novel* all those miRNAs belonging to gene families for which sequence similarity searches of precursors and mature sRNAs yield no significant hits from miRBase.

Possible miRNA:miRNA* duplexes are identified through partial complementarity of reads mapping on the same strand within 500 bases. Genomic sequences surrounding a tentative miRNA:miRNA* duplex are extracted and minimum free energy structures are estimated. Where candidate miRNA:miRNA* read pairs fall in appropriate positions (1 or 2 bp 3' overhangs on the same stem structure) candidates are subjected to further evaluation. A total of 4048 genomic loci passing this filter were present in both biological replicates and were ranked by the arithmetic mean of four parameters: i) Support Vector Machine output value, ii) strand abundance, iii) strand bias and iv) duplex bias (see Methods for a detailed description of statistical parameters), excluding candidates where 2 or more of the parameters took values lower than 0.8. Low quality secondary structures, loci where high proportions of reads derived from the loop region and loci with excessive production of siRNAs from the complementary strand to the prediction were removed manually. After manual filtering 270 miRNA or miRNA-like loci, producing at least one miRNA:miRNA* duplex were retained.

The miRBase directory (www.mirbase.org, Release 18), contains 142 Brachypodium miRNA loci producing 105 distinct mature miRNAs recovered from various recent studies (Baev et al., 2011; Unver and Budak, 2009; Wei et al., 2009; Zhang et al., 2009). Of these, less than one-third (39 of 142) were independently validated. Our predictions included 66 of these loci (see Table 1, 2, 3 and Supplementary Tables 1, 2, 3 online). In addition to those previously deposited MIR genes, 28 new miRNA genes belonging to already known miRNA gene families were identified (see Table 1 and 2

and Supplementary Tables 1 and 2 online). These include one additional member of the *miR159, miR167, miR5163, miR5167 and miR5181* families, two additional members of the *miR166* and *miR395* families, three *miR5174* family members, five *miR156* loci and eleven *miR5185* family precursors. Notably, we did not predict miRNAs belonging to the highly conserved families *miR162*, *miR394*, *miR398*, *miR399* and to five other known families *miR5203*, *miR5202*, *miR5200*, *miR5199*, *miR5176*, that have been experimentally validated in other monocot species. However, our pipeline identified 94 previously undiscovered and potentially Brachypodium specific miRNA whose precursors show no significant similarity to precursor sequences in miRBase (Table 2 and Supplementary Table 2 online). Based on the similarity of mature miRNA sequences, potential candidates were grouped into 90 gene families, of which only 4 have more than one member. For all the predicted foldback structures presented here our pipeline identified both miRNA and miRNA* sequences, reinforcing the validity of the predictions, as suggested by Meyers and collaborators (Meyers et al., 2008). Finally, 82 miRNA genes were considered as siRNA-like miRNA loci since they were characterized by high redundancy (more than 20 hits on the Bd21 genome), low quality of secondary structure and/or high levels of antisense reads (Table 3 and Supplementary Table 3 online).

In order to assess the phylogenetic distribution of miRNAs, not falling in highly conserved families, and to gain indirect support for the nature of these molecules, we compared our data with 99 small RNA libraries from 34 plant species produced by the "Comparative Sequencing of Plant Small RNAs Project" at University of Delaware (http://smallrna.udel.edu). We searched the database for similar reads with up to three mismatches and/or a 2-nt shift either 3' or 5' with respect to our candidates, in order to

account for the high sequence variability that could occur among different species (see Supplementary Figure 3 and 4 online). We classified 22 miRNA sequences as mostly present in monocot species, with more than 80% of the matching reads derived from monocot species. Moreover 105 miRNA sequences may be defined as putatively *Brachypodium* specific, having less than 10 matches in other small RNA libraries.

We noted that members of the conserved families show a consistent pattern of reads clustered almost exclusively at the mature miRNA and its corresponding miRNA* position (Figure 2 A-B). Conversely, for the majority of potentially lineage-specific miRNA loci a more evenly distributed background level of reads mapping to the entire stem region of the precursor is observed - despite the presence of a few highly abundant mature small RNAs (corresponding to miRNA and, often, the miRNA*) (Figure 2 C). Considering the structures of mapped pre-miRNAs and their duplexes it was also possible to identify MIR genes in which more than one distinct miRNA:miRNA* pair are produced from the same precursor sequence (Figure 3). This is the case, for example, of the conserved loci *miR169a*, *miR169j* and *miR319b* where, in addition to the highly expressed miRNA duplex deposited in the public database, a second less abundant miRNA pair was identified. For the new member *miRCB159b*, all three duplexes have a relatively high expression, suggesting an active role of all the three miRNA duplexes in post-transcriptional regulation. Multiple miRNA:miRNA* pairs derived from the same precursor tend to be arranged in a phased manner in accordance with previous observations in Arabidopsis (Bologna et al., 2009) and Physcomitrella (Addo-Quaye et al., 2009). Production of more than one miRNA:miRNA* duplex of either 21 or 24 nucleotides in length is more frequent within the novel and recently emerged miRNAs, where 11 genes exhibit this phenomenon (Table 2 and

Supplementary Table 2 online), again in accordance with the models for the origin and evolution of miRNA precursors (Vazquez, 2006; Vazquez et al., 2010).

Genomic organization of Brachypodium miRNA genes

All except one of the loci of the highly conserved families are annotated as located within intergenic regions, the single exception, *miR166a* corresponds to an intron in an implausible gene model, whereas nearly one fourth of lineage-specific and less deeply conserved loci are positioned within introns of protein coding genes. While an increased density of miRNAs on chromosome 1 and chromosome 3 was reported previously (Baev et al., 2011), we observed no distinct clustering of miRNA genes across the five chromosomes (Figure 4). Notably, for two miRNA genes (*miR5198*, *miR5201*) we confirmed the genomic localization annotated in miRBase, but we propose a new miRNA:miRNA* duplex that corresponds to the most abundant sequences found in our library. Moreover, we identified a novel miRNA (*miRCB185*) located on the opposite strand of the annotated *miR319a* locus.

The two largest MIR families identified in Brachypodium genome are *miR395* (15 genes) and *miR5185* (13 genes) and their members show a degree of pairwise sequence identity in aligned regions ranging from 51.5% to 97% for the *miR5185* family and from 63.2% to 96.7% for the *miR395* family (see Supplementary Figure 2A and 2B online). Ten members of the *miR395* family are clustered on chr5 in only 2 kb, suggesting expansion of the family through tandem duplication - as reported in other plant species and already seen in Brachypodium (Guddeti et al., 2005; Zhang et al., 2009). Our study also considerably expands the lineage-specific *miR5185* family with eleven new precursors, distributed between all five chromosomes. Moreover, due to the higher

abundance in our data of the 3' sequence currently annotated as the *miR5185* star, we define it as the mature miRNA.

Identification of target genes

Plant miRNA targets can often be predicted on the basis of sequence similarity since miRNAs usually show high sequence complementarity to their targets, although such approaches can still produce large numbers of false positive predictions. Two computational programs, psRNAtarget (Dai and Zhao, 2011) and TARGET FINDER (Fahlgren et al., 2007), were used to predict targets for both mature miRNA and miRNA* sequences - a total of 598 small RNAs (497 unique sequences) - as some miRNAs* have been shown to retain a functional role (Devers et al., 2011; Meng et al., 2011; Mi et al., 2008). To increase specificity of predictions, only target predictions provided by both methods were considered (see Supplementary Tables 4, 5 and 6 online). Our conservative analysis allowed the identification of at least one potential target gene for 233 of the miRNA sequences analyzed, corresponding for the most part to the miRNA mature sequences. On average each miRNA sequence has one or two different target genes, with a few exceptions such as the highly conserved *miR156* with 9 target genes all belonging to the Squamosa promoter binding protein-like (SPL) family; the novel *miRCB118* with 12 target genes belonging to different gene families; and *miR5174e-np2* (sequence at the 3' end) with 16 targets, including two Nucleoside Binding Site - Leucine Rich Repeat (NBS-LRR) genes (*Bradi4g03230, Bradi2g38830*) and a Tetraspanin (*Bradi4g30710*), a membrane protein that participates in diverse communication processes, such as cell proliferation, differentiation, and virus and toxin recognition (Cnops et al., 2006; Hemler, 2003).

Conserved miRNAs usually share the same targets across different plant species (Chen, 2009). Indeed, most target predictions for highly conserved miRNAs were as expected from predictions and validations in other species (see Supplementary Tables 4). Most such targets are transcription factors, such as SPL, involved in vegetative phase change; AUXIN RESPONSE FACTOR (ARF), involved in organ elongation; HD-ZIPIII genes, as PHABULOSA (PHB) and REVOLUTA (REV) that play a key role in leaf shape determination and APETALA 2 (AP2), mainly involved in flower development. Some conserved miRNAs target transcripts coding for enzymes such as Sulfate adenylyl transferase (APS4) (*miR395*) and Laccase (LAC) that can be involved in stress response (*miR397). miR168* targets ARGONAUTE 1 (AGO1) and should be involved in an auto-regulatory loop. Overall of the highly conserved miRNAs with well characterized targets, only *miR167* failed to generate the expected target prediction and this was because of a marginally non-significant prediction from one of the tools employed. Some interesting exceptions were found, for example the newly annotated and highly expressed *miRCB159b* was predicted to target a Histidinol-phosphate transaminase, in addition to the conserved targets - MYB65 and MYB33 transcription factors. Among the known and lineage-specific miRNAs a higher proportion of putative targets were related to plant stress/defense response and signal transduction. Among the most highly expressed monocot -specific known miRNAs, *miR528* is potentially involved in the regulation of ethylene degradation through a putative XBAT32 E3 ligase target; *miRCB23a* and *miRCB23b* were predicted to target a protein coding gene (*Bradi3g39450*) containing a WD-40 repeat domain, implicated in a variety of processes including signal transduction, transcriptional control and cell cycle regulation;

miR5168-np2 and *miRCB35* are presumed to regulate the cell cycle by targeting a A-type cyclin and CDC6, respectively.

Expression of miRNAs

The expression levels of both conserved and non-conserved miRNAs vary considerably between our libraries, from highly tissue/condition specific to ubiquitous consistent with accurate dissection of the leaf tissue. To provide an overview of miRNAs expression levels, the sum of the abundances of all eight libraries was calculated and expressed as number of tags per 5 million (TP5M) (Supplementary Table 1, 2, 3 online).

Almost all highly conserved miRNAs have an overall abundance greater than 30 TP5M (90^{th} percentile is ca. 16,000 TP5M) with some, for example *miRNA156* and *miRCB159b,* expressed at more than 16,000 TP5M (see Supplementary Table 1 online). *miRCB159b* whose targets include MYB65 and MYB33 transcription factors and potentially Histidinol-phosphate transaminase, was the most highly expressed miRNA, with 222,381 TP5M.

Putatively novel miRNAs are represented at relatively lower expression levels, ranging from 1 to 12000 TP5M (90^{th} percentile corresponds to ca. 1100 TP5M).

Moreover, we also identified cases in which the 5' and 3' products were both observed at high TP5M (*miR160a, miR166c, miRCB167e, miR168, miR169c, miR171d, miR396e, miRCB88, miRCB141),* an indication of a potential biological role for both of these molecules, as suggested previously (Devers et al., 2011; Meng et al., 2011; Mi et al., 2008). Particular tissue specific trends were observed for families such as *miR156* and *miR396,* where about 93% of reads were observed in the expanding zone (Ec and Es).

The DESeq R/Bioconductor package (Anders and Huber, 2010) was used to gain statistical evidence for differential expression of miRNAs. Four comparisons were performed: drought vs control in the same developmental area and proliferating vs expanding cells in the same growth condition. Using a FDR adjusted *p*-value ≤ 0.2, a total of 40 miRNAs were found to be differentially regulated in the four comparisons.

The first analysis performed addresses miRNA differential expression by leaf developmental stage, evaluating the differential expression between proliferating and expanding cells. In control conditions, three miRNAs are significantly down-regulated in expanding cells (Table 4, Pc vs Ec), with a log2 fold change varying between -3.4 and -4.5. These molecules included two star miRNAs belonging to conserved miRNA families (*miR160* and *miR166*) and the phased 3' product of the *miRCB159b* precursor.

Notably, under drought stress conditions, 32 distinct miRNAs – each with a different sequence – were differentially expressed between proliferating and expanding cells and all were distinct from the three previously mentioned molecules (Table 5, Ps vs Es). Thus, it appears that water shortage modulates miRNAs that may enable the cell to tolerate drought stress. In particular *miR319b* - which targets TCP genes regulating cell proliferation (Palatnik et al., 2003) - is significantly more expressed in proliferating cells than in expanding cells. A regulatory network comprising *miR319*, TCP genes, *miR164* and *miR396* has been described in Arabidopsis (Kim et al., 2003; Rodriguez et al., 2010) and we note a similar correspondence with *miR164*a, *miR164c*, *miR396b* and *miR396e* up-regulated in expanding cells (log2 fold change:4.7, 2.6, 4.4 and 2.5 respectively). *miR396* might target GROWTH REGULATING FACTOR 2 (GRF-2) that governs cell proliferation and expansion (Kim et al., 2003; Rodriguez et al., 2010). Similarly, *miR156* (produced by seven different members of this gene family), is also

up-regulated (log2 fold change 2.8) in expanding cells. In Arabidopsis it has been proposed that cell number and size are regulated by an SPL-dependent pathway involving *miR156* (Usami et al., 2009). Our results suggest that drought stress perturbs the equilibrium between cell size and number, and support the conservation in Brachypodium of the proposed regulatory network.

We directly considered the effect of drought stress in each single cell type. In proliferating cells in control vs stress conditions we identified four miRNAs with significantly changed expression levels (Table 6 Pc vs Ps). All of these miRNAs are down-regulated by drought and are, with the exception of *miR169j,* novel or siRNA-like miRNAs. *miR169j* (log2 fold change -3.1) is predicted, at marginal significance, to target the transcription factor Nuclear factor Y A subunit (NF-YA). In Arabidopsis NF-YA has been demonstrated to be up-regulated by drought stress in a *miR169* dependent manner (Li et al., 2008b). *miRCB22-np2* (log2 fold change of -3.4) is of particular interest, because it is predicted to target a NAC transcription factor, closely related to rice sNAC1, which is known to enhance drought resistance when over-expressed (Hu et al., 2006).

Interestingly, no significant changes were observed in the expanding cells (Ec vs Es) which upon drought show a dramatic size reduction.

Real Time PCR (RT-qPCR) was used to validate the differential expression of 14 miRNAs (*bdi-miR164a*, *bdi-miR164a star*, *bdi-miR528*, *bdi-miR319b-p1*, *bdi-miR167c*, *bdi-miRCB167e star*, *bdi-miR396b*, *bdi-miR396e*, *bdi-miRCB 22-np2 star* ,*miR169j*, *miR5185*, *miR395b*, *miRCB159b* and *miR156b*) identified by the NGS approach (see Tables 4, 5 and 6). Those 14 miRNAs were chosen on the basis of their expression levels, and the biological relevance of putative targets. RT-qPCR was chosen because of

its high sensitivity and specificity in detecting low abundant molecules that allows for discriminating among different members of the same family.

Of 14 selected miRNAs, all except two (*bdi-miR156* and *bdi-miR5185* which show very low melting temperatures) could be detected in cDNA samples from whole young Brachypodium leaf (data not shown). RT-qPCR could produce reliable data only for 8 miRNAs, the remaining four showed either low primer efficiency or melting curves with spurious peaks (data not shown).

Relative abundance of miRNA was estimated in the four samples (Ps, Pc, Es, Ec) using 5.8s rRNA as an internal standard and sample Pc as the reference (see Supplementary Figure 6 online). Fold Change in miRNA expression for the Pc vs Ps; Ec vs Es; Pc vs Ec and Ps vs Es comparisons as estimated from RT q-PCR and sequencing are shown in Figure 5A and 5B.

Trends for the RT-qPCR between expanding and proliferating cells were generally in agreement with the deep sequencing data (Figure 5A) although fold changes were often lower in the PCR experiments. Notably *miR164, miR164-star, miR167, miR167-star, miR396b, miR396e,* and *miR528* are confirmed as more expressed in the Expansion tissue than in Proliferation both in control and stress condition (Es/Ps and Ec/Pc >>1). The comparison between expanding cells under control and stress conditions (Es/Ec) shows a good agreement between the two techniques with stronger and more significant FC revealed by RT-qPCR. *miR396e, miR396b* and *miRCB22-np1* are confirmed to be down regulated in Expanding cells during stress treatment. Interestingly, the *miR319* expression profile inferred from deep sequencing data was never supported by RT-qPCR.

Identification of TAS3 loci in Brachypodium genome

We used the UEA Plant small RNA toolkit facilities (Stocks et al., 2012), with stringent parameters to identify Brachypodium genomic loci producing phased siRNAs in the studied tissues and conditions. Only five genomic loci yielded highly significant scores ($p<0.00005$), and these loci emerged in all experimental conditions and replicates, showing no evidence of differential expression (see Supplementary Figure 5 online). Four of these loci showed high sequence similarity to TAS3 loci in rice, indeed, we were able to identify the expected mir390 target sites flanking the areas of phased siRNA production for each of these loci (See Figure 6) and the most highly conserved regions correspond to the expected mature ta-siRNAs (Adenot et al., 2006). Phylogenetic analyses were consistent with simple orthology relationships with the 4 Rice TAS3 loci annotated in the Cereal Small RNA Database (http://csrdb.ucdavis.edu/cgi-bin/smrna_browse/rice2/). The fifth phased locus corresponds to an annotated NBS-LRR gene. We were not able to confidently identify the small RNA putatively responsible for triggering phased siRNA production at this locus.

Discussion

Plants are able to redistribute resources during adverse conditions. Dissecting the transcriptional and post-transcriptional mechanisms underlying gene expression regulation during stress responses is pivotal to our understanding of how plants adapt to their ever changing environment (Gonzalez et al., 2012).

Here we present a comprehensive characterization of a large set of miRNAs from two different developmental zones (cell proliferation and cell expansion) of the third young

leaf of *Brachypodium distachyon* plants subjected to control and drought stress conditions.

Using NGS technology, eight small RNA libraries were profiled, allowing the confident confirmation of previously deposited miRNAs and the annotation of novel miRNA genes. Moreover, sets of loci regulated in response of drought stress and between the two developing zones of the leaf were identified.

Brachypodium miRNAs

NGS technology has become a preferred approach for small RNA discovery, as the generation of millions of reads allows the detection of even rare small RNAs species (McCormick et al., 2011). Our *ab-initio* bioinformatics pipeline based on the detection of miRNA:miRNA* pairs and accounting for general characteristics of miRNA hairpins processed by DICER LIKE (DCL) enzymes (Meyers et al., 2008) was able to process the complexity of the small RNA populations (Li and Liu, 2011), detecting not only conserved and non-conserved miRNAs, but also siRNA-like miRNAs.

In previous studies 142 miRNA loci producing 105 distinct mature miRNAs were annotated in *Brachypodium distachyon* (Baev et al., 2011; Unver and Budak, 2009; Wei et al., 2009; Zhang et al., 2009). Our data confirmed approximately half (66) of the current Brachypodium miRNA sequences deposited in miRBase (www.mirbase.org) and extended the list of Brachypodium miRNA genes. More specifically, a total of 270 loci, divided in 17 highly conserved, 106 less conserved or novel miRNAs families and 82 siRNA-like miRNAs were identified. We were able to reconstruct precursor sequences for representatives of 17 of the 21 miRNA families recognized to be highly conserved in diverse plant species (Axtell and Bowman, 2008), supporting the

sensitivity of our pipeline. Given that expression of many conserved miRNAs is tissue or environmental condition-specific (Jeong et al., 2011) the observation of expression of all conserved miRNAs in restricted tissue samples is not necessarily expected.

Of the four conserved families for which precursors were not identified in our study, *miR394*, which targets in Arabidopsis an F-box protein involved in leaf morphology (Song et al., 2012), was abundantly expressed, however its miRNA* sequence was not observed in both biological replicates and therefore was discarded by our conservative pipeline.

miR398 and *miR*399, were not detected at all, however this is not surprising since their expression is restricted to specific stress conditions not tested (Aung et al., 2006; Bari et al., 2006; Gu et al., 2010; Sunkar et al., 2006; Zhu et al., 2011). *miR162* targets DCL1, involved in miRNA biogenesis, and only 4 reads corresponding to its mature molecule were identified with no miRNA* reads. In grapevine we observed a very limited expression of *miR162* in young leaf when compared to mature leaf (M.E.Pè and E. Mica, unpublished results). Since we focused our analysis on transcriptionally active and young leaf cells, this observation may suggest that this miRNA is simply not expressed in the tissues studied.

As for the remaining Brachypodium miRNAs annotated on miRBase but not found in our libraries, it is probable that we were not able to identify a candidate hairpin structures because of low expression of some lineage-specific miRNAs under the conditions studied. . However, the possibility that some of the Brachypodium miRNAs annotated in miRBase are false positive cannot be excluded.

It is possible to generate some interesting conclusions regarding the different behavior of known and novel miRNAs. Among the identified novel miRNAs a bias was observed

towards 24-nucleotide long molecules (65% were 23-24 nt long, while 35% were 20-22 nt long). 24 base miRNAs are also common in rice (Wu et al., 2010) where their biogenesis is dependent on DCL3. Interestingly, while rice encodes 2 DCL3 proteins – DCL3A and DCL3B (Kapoor et al., 2008; Wu et al., 2010), Brachypodium encodes an additional DCL3A homolog (Supplementary Figure 7 online). In rice these "long miRNAs" (lmiRNAs) are loaded into AGO4 clade proteins and can mediate genomic methylation both in cis of their loci of origin and in trans, at target loci (Wu et al., 2010). Moreover, non conserved and novel miRNAs are, on average, less abundant than well known and conserved miRNAs and the number and type of small RNAs originating from each precursor more variable than in conserved miRNAs. Our bioinformatics pipeline is able to address the challenge of identifying new lineage-specific MIR genes where these less defined patterns are exhibited while conforming to currently accepted miRNA annotation guidelines (Meyers et al., 2008). Our data support and reinforce the hypothesis of Vazquez and co-workers (Vazquez, 2006; Vazquez et al., 2010) that conserved and evolutionarily ancient MIR genes have evolved by progressive mutations in initially perfect inverted repeats - yielding shorter hairpins with more bulges. This evolutionary process is accompanied by a change in hairpin processing by DCLs, in the size of the miRNAs generated and in the number of miRNA:miRNA* pairs generated from each hairpin. Finally, it is generally accepted that younger and lineage-specific miRNAs might be still without a clear functional role, as functionalization of miRNA occurs in concert with structural evolution.

TAS3 loci in Brachypodium

In plants, the targeting of coding or non-coding transcripts by miRNAs can trigger the RNA-dependent RNA polymerase dependent production of dsRNA from products of transcript cleavage. These dsRNA molecules can then be processed by DCL4 to produce phased small RNAs, that subsequently function in gene silencing and potentially in other processes (Chen et al., 2010). TAS precursors that generate ta-siRNAs were originally characterized in Arabidopsis, although the TAS3 family, for which the production of associated siRNA is triggered by interactions of *miR390* with two target sites, is widely conserved in monocots and dicots. A related mechanism affects many NBS-LRR transcripts in some dicots (Zhai et al., 2011) and recent reports suggest that many hundreds of clustered non-coding loci in the rice genome produce phased siRNAs, particularly in floral tissues (Johnson et al., 2009). By searching for loci that produce siRNAs from both strands in a phased manner, we were able to identify four TAS3 loci that are probably orthologous with their counterparts in rice. None of these loci exhibited differential levels of ta-siRNA production between tissue types or between control and stress conditions. The lack of differential expression is perhaps unsurprising as TAS3 derived ta-siRNAs are implicated in the determination and maintenance of abaxial-adaxial leaf polarity (Garcia et al., 2006), a process that is not obviously affected between drought and control conditions.

Additionally, we identified a single NBS-LRR gene that exhibited significantly phased siRNA production. We were not able to identify in phase target sites either for members of the *miR2118*/*miR482* family implicated in triggering phased siRNA production in monocots (Zhai et al., 2011), or for other miRNAs identified in this study and the identity of the putative trigger molecule at this locus remains unknown.

Roles of miRNAs in leaf growth during standard and water deficit conditions

The expression of a large number of miRNAs in leaves reflects the complexity of regulatory activities required for fine-tuning growth and development of this organ (Johnson and Lenhard, 2011; Kidner, 2010). Several miRNA-target nodes have been described as coordinating gene expression programs to support phenotypic plasticity (Rubio-Somoza and Weigel, 2011). Proximo-distal leaf patterning - defined as the primary axis of growth – is driven by two main processes: proliferation and expansion of cells (Gonzalez et al., 2012). To date pathways controlling cell proliferation in leaves have not been fully described and much less is known about cell expansion (Donnelly et al., 1999). Moreover, how these cell autonomous processes interact and which miRNA-target nodes converge on the regulation of the cell-cycle has not yet been studied in detail.

In this work we exploited the capacity of drought to perturb cell proliferation and/or expansion in order to identify miRNAs regulating leaf development and final leaf size. *miR396,* which targets GROWTH-REGULATING FACTORS (GRFs) an important class of transcriptional regulators involved in the control of cell proliferation in leaves (Kim et al., 2003), appears to play a central role in our Brachypodium system. RT-qPCR and NGS data show strong up-regulation of *miR396b and miR396e* during leaf development in Brachypodium and it is reasonable to hypothesize that, as in Arabidopsis, *miR396* restricts GRF expression contributing to proliferation arrest in expanding cells (Rodriguez et al., 2010). Recent studies imply an intriguing regulatory network between *miR164*, *miR396* and *miR319* in Arabidopsis: *miR319* regulates five members of the TCP transcription factor family (TCP2, 3, 4, 10, 24) that function as inhibitors of cell proliferation and directly bind *miR164a* promoter region (Martin-Trillo and Cubas, 2010; Palatnik et al., 2003) In addition, it has been shown that a point

mutation in the *miR319* target site of TCP4 leads to accumulation of *miR396*, lower GRF transcript levels and the formation of smaller leaves (Rodriguez et al. 2010). The inverse relationship between *miR319* and *miR164/miR396* expression level is supported, in Brachypodium, by our sequencing data. Consistent with a central role for *miR164* in the transition between cell proliferation and expansion, *miR164* targets NAC transcription factors, such as CUC, which play an important role in the formation of shoot meristems in leaf axils (Hasson et al., 2011; Koyama et al., 2010; Vroemen et al., 2003). Moreover our data suggest that the *miR164a* star sequence, which is also up-regulated in expanding cells (and confirmed by RT-qPCR validation), targets the transcript of a *TCP* gene (*Bradi3g59320*), hinting at possible feedback regulation of *miR164*. Taken together, these results support the conservation in monocots of an miRNA regulatory network, composed of three miRNA-target nodes: *miR319*-TCP, *miR164*-NAC, *miR396*-GRF, shown to be involved in the control of cell proliferation during the early phases of Arabidopsis leaf growth (Rubio-Somoza and Weigel, 2011).

Another interesting miRNA-mediated network influencing leaf size centers on *miR156*. In Arabidopsis the mutation *more and smaller cells 1*, determining bigger leaves with an increased cell number and decreased cell size, is due to a reduced sensitivity to *miR156*-driven cleavage of the *SPL15* gene transcript (Usami et al., 2009). In Brachypodium we observed up-regulation of *miR156* (log2 fold change 2.8) in expanding cells under drought stress. This suggests that drought stress perturbs the normal equilibrium between cell size and number, and is consistent with a SPL-dependent drought response pathway involving *miR156* in monocots.

Particular attention should be paid to the monocot specific miRNA *miR528* found to be up-regulated in expanding cells during drought stress (data from deep sequencing

confirmed by RT-qPCR). Our observation confirms previous data in Sugarcane (Ferreira et al., 2012) and Brachypodium (Budak and Akpinar, 2011) where drought stress, similarly, up-regulates *miR528*. While different targets have been proposed for *miR528*, in Brachypodium it is predicted to target a homolog of XBAT32, a RING type E3 ligase (Prasad and Stone, 2010). In Arabidopsis, *xbat32* mutants exhibit ethylene overproduction when compared to the wild type (Prasad et al., 2010). It is known that ethylene signaling plays a role in mediating abiotic stress responses including to drought (Kalantari et al., 2000; Sobeih et al., 2004). Furthermore, drought stress causes ethylene mediated cell cycle arrest in developing leaves (Skirycz et al., 2011). The regulation of *XBAT32* by *miR528* might thus act to fine tune growth responses during hydric stress.

The *miRCB167e* locus may also possess particular significance. The 3' star RNA is unique, among the *miR167* family, in being expressed at comparable levels to the mature miRNA with both deep sequencing and RT-qPCR indicating strong up regulation in expanding cells under drought stress. The predicted target is an UDP Glucose 6 Dehydrogenase (*Bradi4g25140*), a key enzyme in cell wall biosynthesis expressed in growing tissues (Karkonen and Fry, 2006; Klinghammer and Tenhaken, 2007). These data suggest an interesting additional function in leaf development and cell expansion for the *miR167e* gene.

Conserved canonical targets for *miR159* include *GAMYB-like* genes (i.e. MYB33 and MYB65) implicated in gibberellin (GA) signaling in anthers and germinating seeds (Achard et al., 2004; Alonso-Peral et al., 2010). However, in tomato a new *miR159* target of unknown function, was recently identified and its accumulation in leaves was demonstrated when miRNA-mediated cleavage was inhibited (Buxdorf et al., 2010). An

additional non canonical target, Histidinol-phosphate transaminase, was predicted for *miRCB159b,* the most abundant miRNA in all eight libraries (relative abundance > 222000TP5M) in Brachypodium. Although the tomato target is not homologous to our non canonical predicted target these data testify to the tendency of *miR159* to assume new functions.

The role of *miR169* in drought tolerance is controversial; it was recently shown in Arabidopsis that the transcription factor NF-YA5 is targeted by *miR169* and that over-expression of *miR169* leads to water loss and hypersensitivity to drought stress (Li et al., 2008b). By contrast, in tomato it was demonstrated that drought stress induces *miR169,* enhancing drought tolerance and that plants over-expressing *miR169c* displayed reduced stomatal opening and lowered leaf water loss (Zhang et al., 2011). Our data are in agreement with the findings in Arabidopsis, showing a down-regulation of *miR169j* in the proliferating zone under stress and support the role of *miR169* in the post-transcriptional regulation of NF-YA.

Finally, the newly identified *miRCB22-np2* is of some interest as it is down-regulated under drought conditions in proliferating cells and also to some extent in expanding cells (although we were unable to confirm the latter through RT-qPCR). Its predicted target is a NAC transcription factor, highly similar to the rice sNAC1. Over-expression of sNAC1 enhances drought tolerance though increased stomatal closure and/or ABA sensitivity to prevent water loss (Hu et al., 2006).

On the whole, the work presented here represents a considerable increase in our knowledge of miRNAs encoded in the genome of *Brachypodium distachyon*. Furthermore for the first time we show differences in expression profiles of miRNAs between proliferating leaf cells and cells undergoing expansion in both normal and

drought stressed conditions, emphasizing the importance of the study of individual cell types to gain insight into finely regulated processes during abiotic stress responses. The data obtained represent a critical first step towards the dissection of the impact of small RNA-mediated processes on growth and development during hydric stress in Brachypodium. Differential expression analyses and *in silico* target predictions allow the formation of specific and testable hypotheses regarding the existence and potential importance of both novel and conserved miRNA target pairs and regulatory nodes. Such hypotheses are the subject of ongoing experimental work and are expected to lead to further insights into lineage-specific and widely conserved responses to drought stress; one of the most relevant objectives in modern plant biology.

Methods

Plant material and leaf sampling for molecular analysis

All samples were recovered from the third leaf (leaf 3) of *Brachypodium distachyon* inbred line 21 (Bd21), Plants were harvested at a fixed time point in the afternoon, about 24 hours after the emergence of leaf 3. The growing leaf 3, between 1.5 and 2 cm in size at that point, was carefully removed from the leaf sheath of the older two leaves, without damaging the fragile meristem at its base. Samples were immediately stored in RNA-Later solution (Ambion, Austin, Texas). After an overnight incubation at 4°C leaves were dissected into three distinct developmental zones. Based on microscopic observations we defined the proliferation zone as the first 2 mm from the leaf base, the expansion zone as the next 4 mm, and the mature zone as the remaining distal part of

the leaf. All the collected leaf zones were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Drought stress treatment

Bd21 drought stress treatment was identical to that described in Verelst et al., (2012). Briefly, lemma and palea, the two seed coats, were removed prior to sowing. Seeds were sown in Jiffy soil in a large Petri dish and incubated at 4°C for 3 days, in darkness. Brachypodium plants were germinated and grown in a growth chamber under conditions of 16 hours of light, at 24°C and 55% relative humidity. After three days, when all seeds had germinated synchronously, individual plantlets, comprising only first leaf, were carefully transferred to pots (5.2 cm diameter; 5.2 cm high), containing an equal amount of soil (technic5, Free Peat, NL). At the time of plantlet transfer all pots contained 2.27 g water per gram of dry soil. Pots containing control plants were dried down to 1.82 g g-1, while pots containing plants that would be subjected to drought stress were dried down to 0.45 g g^{-1} (severe drought stress).

All plants grew synchronously, and the 3rd leaf of plants within the same experiment always appeared within a 24-hour time window.

Construction and sequencing of small RNA libraries

Small RNA fractions were extracted using mirPremier™ kit (Sigma) according to manufacturer's protocols from proliferating and expanding leaf zones grown under stress and control conditions. Small RNA quality and quantity was evaluated with NanoDrop Spectrometer (ND 1000, Celbio SpA) and by running an aliquot on 3% agarose gel.

Eight small RNA libraries (4 tissues and 2 biological replicates), were prepared using TruSeq Small RNA sample preparation kit, Illumina Inc (CA, USA). Briefly, RNA adaptors were ligated to the 5' and 3' ends of the small RNA molecules, followed by reverse transcription and 15 cycles of PCR amplification. Library quality was assessed on the Agilent 2100 Bioanalyzer (Agilent Technologies) cDNA libraries were loaded on the Cluster Station and individually sequenced at ultra-high throughput on Illumina Genome Analyzer II. Sequencing was performed at Applied Genomics Institute (IGA), Udine, Italy.

Our raw data have been submitted to SRA Archive with the following id: Submission: SRA055941/Brachypodium_leaf_drought_stress Experiments: SRX160390-SRX160397

Bioinformatics analyses

Adapter sequences were trimmed from small RNA reads using a custom python script that allowed up to two mismatches with the adapter sequence in order to accommodate the known reduction of sequence quality in the 3' part of Illumina reads. Trimmed small RNA deep sequencing reads were mapped to the reference Brachypodium genome (Bd21) using the SOAP program (Li et al., 2008a; Li et al., 2009). Reads that perfectly match the Bd21 genome, excluding those matching to structural RNAs (tRNA and rRNA), were retained and used for further analysis.

Data processing was accomplished using custom Python and Perl scripts developed by us (D Horner, unpublished results). An *ad-hoc* miRNA identification pipeline, based on the properties of known plant miRNAs and their precursors (Meyers et al., 2008), was

used. In brief the read map of each chromosome is scanned in a 5'- 3' direction independently for each DNA strand.

1) All pairs of non overlapping reads are subjected to a simple test to exclude pairs that cannot represent or approximate miRNA:miRNA* sequence pairs. This is achieved by reversing the sequence of the 3' mapped read and creating fixed alignments of with from 4 to zero base 3' overhangs. For each such alignment, the maximum number of contiguous complementary (C/G, A/T, G/T) pairs is counted. If no stretch of 5 or more contiguous complementary bases (the minimum number observed in experimentally validated plant miRNA:miRNA* pairs) is detected, the pair is discarded. If the pair of reads represents a "potential" miRNA:miRNA* pair, the genomic coordinates and frequencies of occurrence of each of the reads are recorded and a "tentative precursor" is defined as the region from ten bases upstream of the 5' read to 10 bases downstream of the 3' end of the downstream read.

2) At the end of this initial genomic scan, all "tentative precursors" that have start and stop coordinates shifted by less than 6 positions, and are strictly nested one within the other, are merged and information regarding associated reads is retained

3) RNAfold software from the Vienna package (Hofacker et al., 1994) is used to estimate minimum free energy structures for all merged tentative precursors and to test whether associated reads map onto opposing arms of single stems and that at least one associated read pair provides the expected 2 base 3' overhangs expected of miRNA:miRNA* pairs on the predicted structure.

4) The read-associated stems of tentative precursors passing these criteria are subjected to evaluation by a Support Vector Machine (SVM), based on that proposed by (Xue et al., 2005), but also including 50 additional features describing sequence,

structural and thermodynamic properties of the candidate precursor. This system utilized functions from the open source library LibSVM (Chih-Chung and Chih-Jen) and was previously shown to be both sensitive and specific (V.Piccolo and D.S.Horner, unpublished results). A probability score that the structure provided could represent a real miRNA precursor is generated by the SVM and recorded. All reads mapping each tentative precursor locus (on both strands) are recovered from the original read mapping files and used to calculate three *ad-hoc* statistics that are designed to quantify expected characteristics of small RNAs derived from genuine miRNA precursors, as indicated below.

Strand abundance, the proportion of all reads mapped to the precursor locus that derive from the strand predicted to encode the precursor (a high value is consistent with a single strand RNA biogenesis mechanism). *Duplex bias*, the proportion of all reads mapping to the candidate precursor region, on the expected strand, that are involved in canonical miRNA:miRNA* interactions (2 base 3' overhangs). For cleanly processed miRNA precursors, this value is expected to approach 1. *Strand bias*, 1 minus the proportion of sites in the tentative precursor locus that account for 90% of all reads (numerically) that map to the precursor locus. Again, for a true miRNA precursor, the majority of reads are expected to be miRNA and miRNA*. The closer that this expectation is fulfilled, the closer to 1 the final statistic will be.

A final statistic is calculated as the mean of the three read mapping statistics and the probability score for the precursor generated by the SVM. Candidate precursors are ranked according to this statistics and then manually examined. We considered putative miRNAs all the structures whose mean value is comprised between 1 and 0.65.

Sequence similarity searches were performed against miRBase to identify homologues of known miRNA families from Brachypodium and/or other plant species.

The linear count scaling method TP5M were chosen for data comparison according to the following formula: normalized abundance (TPM) = raw abundance/ (total genome match – t/r/sn/snoRNA/chloroplast/mitochondria)/total reads in library × 5,000,000.

Phased siRNA analyses were performed using the ta-siRNA prediction tool in the UEA sRNA workbench (Stocks et al., 2012) with default parameters.

The chromosome distribution charts were created using the ggbio tool, an R/Bioconductor package (Yin et al., 2012).

DCL family protein sequences were identified by similarity searches at phytozome.net and aligned using the software muscle (Edgar, 2004). Unambiguously aligned regions were excluded using GBlocks (Talavera and Castresana, 2007). Neighbor joining trees with 100 bootstrap replicates were generated using PHYLIP (Felsenstein, 1993).

Conservation of the miRNA candidates was evaluated by a search across 99 small RNA libraries from 34 plant species (GEO Series GSE28755) including green algae, ferns, gymnosperms, dicots and monocots. All the small RNA reads in range of 20 to 24 in size and represented by at least two reads in a library were aligned to the miRNA candidates. For the screening, we followed the procedure described in (Sato et al., 2012); briefly a maximum of 3 mismatches was allowed and up to 2 nt overhanging nucleotides at the 5' and/or 3' end. Alignments were performed using SeqMap (Jiang and Wong, 2008). Sum of abundances of all the variants identified per each miRNA candidate were reported for each small RNA library (see Supplementary Figure 3 online).

miRNA Target prediction

All miRNAs and siRNA-like miRNA loci here described were used to predict targets from the transcript database generated by JGI v1.0 8x assembly of *Brachypodium distachyon* Bd21 and the MIPS/JGI v1.0 annotation (Vogel et al., 2010). Two approaches were applied to identify targets for the candidate miRNAs: psRNATarget (www.plantgrn.org/psRNATarget/) (Dai and Zhao, 2011), and TARGET FINDER release 1.6 (http://www.carringtonlab.org/resources/targetfinder), (Fahlgren et al., 2007). psRNATarget evaluates complementarity between the small RNA and its target gene using Smith–Waterman implementation, ssearch (Version 36.x) plus a target-site accessibility evaluation by calculating energy required to open the secondary structure around a small RNA target site on the mRNA. TARGET FINDER has been developed using a FASTA search algorithm, scoring the results based on position and frequency of the mispaired nucleotide and calculating Maximum Free Energy of the miRNA/target pair. Analyses were done using the following score cutoff: 2.5 for psRNA Target (where the default parameter is set to 3) and 4 (the suggested score cutoff) for TARGET FINDER selecting only those targets that were identified by both algorithms, in order to reduce false positive rate.

Differential expression analysis of miRNAs under drought stress

Differential expression analysis were performed using DESeq (Anders and Huber, 2010) a package for the statistical environment R and distributed within the Bioconductor project (Gentleman et al., 2004). This tool is based on the negative binomial distribution (NB), and is well suited to data from experiments with small numbers of biological replicates due to the better estimation of the raw variance.

To identify differentially expressed loci, we start from 57500 sequences originating from 4048 hairpins identified in both replicates, having at least a total of 10 reads in all the eight libraries. A *p*-value ≤ 0.01 and a relaxed FDR cutoff ≤ 0.2 were applied. Differentially expressed miRNAs during drought/control condition and during leaf development were investigated.

RealTime PCR on mature miRNAs

Total RNA was extracted from leaf tissues, pooling at least 90 plants using same leaf sections as those used for small RNA libraries preparation) using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich), and treated with RNase-free DNAseI (Sigma-Aldrich). Total RNA was quality checked and the absence of genomic DNA was assessed performing a control PCR on the Ubiquitin gene (Bradi1g32860.2) whose forward primer is designed on the second intron. Treated total RNA (2 μg) was subjected to poly-adenylation and reverse transcription using the miScript II RT Kit (Qiagen),following manufacturer's directions and using the miScript HiSpec buffer provided.

RT-qPCR was performed using the miScript SYBR® Green PCR Kit (Qiagen), assembling each reaction in 15 μl having three technical replicates and running each plate on the BioRad CFX96 Real Time System (BioRad). Fourteen different miRNAs were tested and 5.8s ribosomal RNA was used as an internal standard (Shi and Chiang, 2005; Xue et al., 2009). The reverse primer used is the Universal Primer provided with the kit, while forward primers correspond each to the entire sequence of the miRNAs tested; all primers used are listed in Supplemetary Table 8 online. Reaction efficiencies of RT-qPCR assays for each individual miRNA/primer were determined using a 4-fold dilution series of leaf cDNA and generating a standard curve plotting the cDNA

concentration versus the corresponding Ct (Treshold cycle). Efficiency was calculated from the slope of the standard curve, using the BioRad CFX Manager Software (See Supplementary Table 8 online). Relative quantification of each miRNA tested was calculated from Ct value, using the $2^{-\Delta\Delta Ct}$ method, directly with the BioRad CFX Manager Software.

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Supplementary data

Supplementary Figure 1 online: Mean redundancy of small RNA reads by length

Supplementary Figure 2 online: Sequences alignment of the miRNA precursors of miR395 and miR5185 families.

Supplementary Figure 3 online: Abundance of each Brachypodium miRNAs in small RNA libraries from various plants and green algae deposited at http://smallrna.udel.edu

Supplementary Figure 4 online: Pie chart representing the comparative analysis between our Small RNAs sequences and the data produced by the "Comparative Sequencing of Plant Small RNAs Project".

Supplementary Figure 5 online: 5 phased loci in Brachypodium genome.

Supplementary Figure 6 online: RT-qPCR quantification of Brachypodium miRNAs.

Supplementary Figure 7 online: Phylogenetic tree of DCL sequences from Brachypodium and Rice.

Supplementary Figure 8 online: Mapping of small RNA reads to predicted miRNA and miRNA-like precursors

Supplementary Table 1 online: *Brachypodium distachyon* conserved microRNAs.

Supplementary Table 2 online: *Brachypodium distachyon* novel microRNAs.

Supplementary Table 3 online: *Brachypodium distachyon* si-RNA like microRNAs.

Supplementary Table 4 online: Putative targets of *Brachypodium distachyon* conserved microRNAs.

Supplementary Table 5 online: Putative targets of *Brachypodium distachyon* novel microRNAs.

Supplementary Table 6 online: Putative targets of *Brachypodium distachyon* si-RNA like microRNAs.

Supplementary Table 7 online: Summary Statistics of deep-sequenced small RNAs libraries.

Supplementary Table 8 online: Primer sequences used for RT-qPCR.

Figure Legends

Figure 1: Counts (in millions) of total mapped small RNA reads (tRNAs and rRNAs were removed), ranging between 18 and 26 nt in length, in the four different small RNA libraries sequenced. The average between the two biological replicates is plotted for each condition. Ps: Proliferating cells under drought stress (21nt:1,254,013(11.80 %) – 24nt:6,911,678(65.03 %)); Pc: Proliferating cells under control conditions (21nt:2,046,801(12.25 %) – 24nt:1,126,630(66.58 %)); Es: Expanding cells under drought stress (21nt:2,072,160(10.96 %) – 24nt:13,162,775(69.61 %)); Ec: Expanding cells under control conditions (21nt: 2,096,772(12.97 %) – 24nt:10,454,600(64.65 %)).

Figure 2: Small RNA reads plot for selected miRNA precursors. The sum of frequencies of small RNAs in all the libraries (Pc, Ps, Ec, Es) are plotted by position on the indicated miRNA precursors. 2A, 2B: Conserved families show a clear pattern of reads mapped to the stem region of the precursor. 2C: Lineage-specific miRNAs show an evenly distributed reads mapping to their precursor. miRNA (m) and miRNA star (*) are indicated. m-a and m-b indicate two distinct mature miRNAs (miRCB22-np1 and miRCB22-np2) originating from the same precursor, in this case star sequences are not shown.

Figure 3: Examples of miRNA loci generating more than one distinct miRNA:miRNA* pair from the same precursor. Six loci are presented: *miRCB159b*, *miR169a*, *miR169j*, *mir319b*, *miR5168* and *miR5178*. For each precursor: MIR locus, mapping position (Chromosome, strand, coordinates), Free Energy (ΔG) of the secondary structure, Sum of the abundance (TP5M) of the most frequent tag (highlighted), are presented along with the precursor sequence and dot-bracket notation representing RNA stem-loop structure. Below the dot-bracket notation distinct miRNA:miRNA* pairs are aligned. Notably, for miR169j the conserved mature sequence is less abundant than its star sequence.

Figure 4: Chromosome distribution of miRNA genes in the *Brachypodium distachyon* genome. A) Known microRNAs; B) Novel miRNAs; C) si-RNA like microRNAs. MIR genes are plotted on the circular representation of the Brachypodium genome using the coordinates of their predicted pre-miRNA and shown as green bars.

Figure 5: Expression Fold Change of different miRNAs among two experimental conditions. 5A: Expanding vs Proliferating cells in either control or stress conditions. For each miRNAs the fold changes resulting from deep sequencing data (NGS) and from Real Time approach (RT-qPCR) are shown, in order to directly compare the two techniques. 5B: Stress vs Control in either Proliferating or Expanding cells. For each miRNAs the fold changes resulting from deep sequencing data (NGS) and from Real Time approach (RT-qPCR) are shown, in order to directly compare the two techniques. Error bars relative to RT-qPCR experiments represent the estimate of standard error of the mean (SEM) for the three replicates, as calculated from the BioRad Software. A logarithmic scale is used along the Y axis.

Figure 6: Muscle alignment of Rice, Arabidopsis TAS3 loci and the 4 identified Brachypodium TAS3 loci. 3' and 5' miR390 target sites are shown. Highlighted red boxes represent the most highly conserved regions corresponding to the expected mature ta-siRNAs from Rice and Arabidopsis.

Table 1: List of conserved *B. distachyon* microRNAs and their genomic coordinates on the Bd21 genome JGI v1.0 8x. A name has been given to each miRNA sequence according to miRBase Release 18 and for the newly discovered miRNAs a provisional name has been given using a sequential numbering associated with the abbreviation miRCB (miRNA Candidate Brachypodium). p1, p2 refer to phased siRNA produced

from the same hairpin; np1, np2 refer to different siRNA produced from the same hairpin. S: strand. nt: sequence length of the microRNA.

Table 2: List of novel *B. distachyon* microRNAs and their genomic coordinates according to the Bd21 genome JGI v1.0 8x. For the newly discovered miRNAs a provisional name has been given using a sequential numbering associated with the abbreviation mirCB (miRNA Candidate Brachypodium). p1, p2 refer to phased siRNA produced from the same hairpin; np1, np2 refer to different siRNA produced from the same hairpin. S: strand. nt: sequence length of the microRNA.

Table 3: List of si-RNA like *B. distachyon* microRNAs and their genomic coordinates according to the Bd21 genome JGI v1.0 8x. For the newly discovered miRNAs a provisional name has been given using a sequential numbering associated with the abbreviation mirCB (miRNA Candidate Brachypodium). p1, p2 refer to phased siRNA produced from the same hairpin; np1, np2 refer to different siRNA produced from the same hairpin. S: strand. nt: sequence length of the microRNA.

Table 4: List of microRNAs showing a differential expression depending on developmental area, in control condition (Proliferating cells Vs Expanding cells). Pc: Proliferating cells in control conditions; Ec: Expanding cells in control conditions. FDR: False Discovery Rate (adjusted *p*-value). Target: tentative target as predicted by our stringent pipeline. *: Targets predicted only by one of the two Software tools used (psRNAtarget or TARGET FINDER) and at marginal significance. N.A. no target was identified.

Table 5: List of microRNAs showing a differential expression depending on developmental area under stress condition, (Proliferating cells Vs Expanding cells). Ps: Proliferating cells under severe drought stress; Es: Expanding cells under severe drought stress. FDR: False Discovery Rate (adjusted *p*-value). Target: tentative target as predicted by our stringent pipeline. *: Targets predicted only by one of the two Software tools used (psRNAtarget or TARGET FINDER) and at marginal significance. N.A. no target was identified.

Table 6: Differentially expressed microRNAs depending on environmental condition (drought Vs control condition) in proliferating cells. Pc: Proliferating cells in control conditions; Ps: Proliferating cells under severe drought stress. FDR: False Discovery

Rate (adjusted *p*-value). Target: tentative target as predicted by our stringent pipeline. *: Targets predicted only by one of the two Software tools used (psRNAtarget or TARGET FINDER) and at marginal significance. N.A. no target was identified.

Table 1: *Brachypodium distachyon* known microRNAs

miRNA name	Genomic localization	S	miRNA 5p	nt	miRNA 3p	nt	miRNA name	Genomic localization	S	miRNA 5p	nt	miRNA 3p	nt
miR156b	Bd3:39258130:39258298	-	UGACAGAAGAGAGUGAGCAC	20	GCUCACUUCUCUCUCUGUCACC	22	miR395j	Bd5:25456154:25456239	+	GUUUCCCGCAAGCACUUCACG	21	UGAAGUGUUUGGGGGAACUC	20
miR156d	Bd5:18202201:18202314	-	UGACAGAAGAGAGUGAGCAC	20	GCUCACUGCUCUGUCUGUCACC	22	miR395k	Bd5:25456288:25456378	+	GUUCCCUGCAAGCACUUCAUG	21	UGAAGUGUUUGGGGGAACUC	20
miRCB156e	Bd2:4030451:4030583	+	UGACAGAAGAGAGUGAGCAC	20	GCUCACUUCUCUCUCUGUCAGC	22	miR395l	Bd5:25456565:25456655	+	GUUCCCUGCAAGCACUUCACG	21	UGAAGUGUUUGGGGGAACUC	20
miRCB156f	Bd2:4030679:4030788	+	UGACAGAAGAGAGUGAGCAC	20	GCUCACUGCUCUAUCUGUCAGC	22	miR395n	Bd5:25457116:25457205	+	GUUCCCUGCAAGCACUUCACC	21	UGAAGUGUUUGGGGGAACUC	20
miRCB156g	Bd2:4030916:4031026	+	UGACAGAAGAGAGUGAGCAC	20	GCUCACCCUCUCUCUGUCAGC	21	miRCB395o	Bd1:55440734:55440819	-	AUUCCCUACAAGCACUUCACA	21	UGAAGUGUUUGGGGGAACUC	20
miRCB156h	Bd3:49973597:49973704	-	UGACAGAAGAGAGUGAGCAC	20	GCUCACUGCUCUUCCUGUCAUC	22	miRCB395p	Bd5:25456430:25456518	+	GUUUCCUGCAAGCACUUCACG	21	UGAAGUGUUUGGAGGAACUC	20
miRCB156i	Bd4:36168961:36169114	+	UGACAGAAGAGAGUGAGCAC	20	GCUCACCCUCUCUCUGUCAGC	21	miR396a	Bd3:59349803:59349973	-	UCCACAGGCUUUCUUGAACUG	21	GUUCAAGAAAGUCCUUGGAAA	21
miR159	Bd2:5602239:5602433	+	AGCUCCCUUCGAUCCAAUC	19	CUUGGAUUGAAGGGAGCUCU	20	miR396b	Bd5:27112481:27112632	+	UCCACAGGCUUUCUUGAACUG	21	GUUCAAGAAAGCCCAUGGAAA	21
miRCB159b-p1	Bd2:1091342:1091539	+	GAGCUCCUAUCAUUCCAAUGA	21	UUUGGAUUGAAGGGAGCUCUG	21	miR396c	Bd3:54968158:54968269	-	UUCCACAGCUUUCUUGAACUG	21	GUUCAAUAAAGCUGUGGGAAA	21
miRCB159b-p2	Bd2:1091342:1091539	+	AAGGUCUGUCAGAAGGGUGAUAC	23	AUCCACCCCUUGCCGACCGCUG	22	miR396d	Bd1:46677024:46677134	-	UUCCACAGCUUUCUUGAACU	20	UUCAAUAAAGCUGUGGGAAA	20
miRCB159b-p3	Bd2:1091342:1091539	+	AGCUGCUUGUUCAUGGUUCCC	21	UUUGCAUGACCGAGGAGCCGC	21	miR396e	Bd3:54962849:54963022	+	UUCCACAGCUUUCUUGAACUU	21	GGUCAAGAAAGCUGUGGGAAG	21
miR160a	Bd1:4550723:4550858	-	UGCCUGGCUCCCUGUAUGCCA	21	GCGUGCGAGGAGCCAAGCAUG	21	miR397b	Bd3:3177780:3177881	-	AUUGAGUGCAGCGUUGAUGAA	21	CUUCGACCCUGCACCCAAUCA	21
miR160b	Bd1:28020431:28020539	-	UGCCUGGCUCCCUGUAUGCCA	21	GCGUGCAAGGAGCCAAGCAUG	21	miR408	Bd2:10450365:10450514	+	CAGGGAUGGAGCAGAGCAUG	20	CUGCACUGCCUCUUCCCUGGC	21
miR160c	Bd3:3414595:3414705	-	UGCCUGGCUCCCUGUAUGCCA	21	GCGUGCAAGGAGCCAAGCAUG	21	miR529	Bd3:44898515:44898610	+	AGAAGAGAGAGAGUACAGCCU	21	GCUGUACCCUCUCUCUUCUUC	21
miR160d	Bd3:12734312:12734424	+	UGCCUGGCUCCCUGUAUGCCA	21	GCGUGCACGGAGCCAAGCAUA	21	miR827	Bd5:18037501:18037625	-	UUUUGUUGGUUGUCAUCUAACC	22	UUAGAUGACCAUCAGCAAACA	21
miR160e	Bd3:41554304:41554409	+	UGCCUGGCUCCCUGAAUGCCA	21	GCAUUGAGGGAGUCAUGCAGG	21	miR528	Bd1:73059564:73059676	-	UGGAAGGGGCAUGCAGAGGAG	21	CCUGUGCCUGCCUCUUCCAUU	21
miR164a	Bd2:19949336:19949504	-	UGGAGAAGCAGGGCACGUGCA	21	CAUGUGCCCUUCUUCUCCACC	21	miR1878	Bd4:37301955:37302059	+	ACUUAGUCUGAACACUAUAAAAAA	24	AUUUGUAGUGUUCAGAUUGAGUUU	24
miR164c	Bd3:59107095:59107193	-	UGGAGAAGCAGGGCACGUGCU	21	CAUGUGCGCUCCUUCUCCAGC	21	miR5163	Bd4:11947866:11947968	-	CCUAGCCUAAAAUAUUUAAAA	21	UUAGGUAUUUCAGGUUAGGUG	21
miR166a	Bd1:6574383:6574491	+	GAAUGACGCCGGGUCUGAAAG	21	UCGGACCAGGCUUCAUUCCCC	21	miRCB5163-b	Bd4:11948702:11948890	-	CACCCAACUGAAAUAUUUAAA	21	UAGAUAUUUCAGGUUGUGUGGA	22
miR166b	Bd1:30655638:30655848	-	GGAAUGUUGUCUGGCUCGGGG	21	UCGGACCAGGCUUCAUUCCCC	21	miR5165	Bd4:33186176:33186378	-	AUCUUGGGCUCUAGGUAGGUU	21	CCUACCUUGAGCCAAAGAUAU	21
miR166c	Bd3:33098780:33098919	+	GGAAUGUUGUCUGGUUCAAGG	21	UCGGACCAGGCUUCAUUCCCC	21	miR5167-p1	Bd4:42126798:42127049	-	CCACUUUGGGUGUCAUUGGUA	21	CCAAUGACACCCAUAGUGGAA	21
miR166d	Bd1:71419399:71419523	-	GGAAUGUUGUCUGGUUGGAGA	21	UCGGACCAGGCUUCAUUCCCC	21	miR5167-p2	Bd4:42126798:42127049	-	UCUAGGUAAGGUAAUUCAAUU	21	GUGAAUUACCUUAACUAGAGC	21
miR166e	Bd3:51437885:51437998	+	GGAAUGUUGUCUGGCUCGAGG	21	UCGGACCAGGCUUCAUUCCCC	21	miRCB5167b	Bd4:42129670:42129875	-	UCUAGUUAAGGUAAUUUAACU	21	UGUGAAUUGCCUUAACGAGAGC	22
miRCB166f	Bd3:57427334:57427473	+	GGUUUGUUGUCUGGCUCGGGG	21	UCGGACCAGGCUUCAAUCCCU	21	miR5168-np1	Bd4:37627842:37628042	+	UCUGGUUCAAGGUCUCCACAU	21	UGUGGUGAUCUCGGACCAGGC	21
miRCB166g	Bd3:27457696:27457817	-	GGCAUGUCGUGUGGCCCGAGA	21	UCGGACCAGGCUUCAUUCCCC	21	miR5168-np2	Bd4:37627842:37628042	+	GGGUUGUUGUCUGGUUCAAGG	21	UCGGACCAGGCUUCGAUCCCU	21
miR167c	Bd1:54067090:54067215	+	UGAAGCUGCCAGCAUGAUCUG	21	GAUCAUGCUGUGCAGUUUCAUC	22	miR5172	Bd1:44311588:44311939	+	CGAGGAGCUAGUAGAUCGGGA	21	CUGAUCUACUAGCUCCUCGGC	21

miRNA name	Genomic localization	S	miRNA 5p	nt	miRNA 3p	nt	miRNA name	Genomic localization	S	miRNA 5p	nt	miRNA 3p	nt
miR167d	Bd3:3632425:3632597	-	UGAAGCUGCCAGCAUGAUCUGA	22	AGAUCAUGUUGCAGCUUCAC	20	miR5173	Bd1:22135912:22136060	-	UCUCGUAUAUGCGGAUGUACC	21	UGCAUCUGUAUAUACGAGAAG	21
miRCB167e	Bd4:1641440:1641565	-	UGAAGCUGCCAGCAUGAUCUGA	22	AGGUCAUGCUGGAGUUUCAUC	21	miR5174e-np2	Bd3:37067795:37067905	+	UACUCCCUCUGUUCCAUAAAG	21	UUUAUGGAACGGAGGGAGUAG	21
miR168	Bd3:1774726:1774816	-	UCGCUUGGUGCAGAUCGGGAC	21	CCCGCCUUGCACCAAGUGAAU	21	miR5174e-np1	Bd3:37067795:37067905	+	CCUCUGUUCCAUAAAGAUUGG	21	CAACCUUUAUGGAACGGAGGG	21
miR169a-p1	Bd2:7704151:7704295	+	CAGCCAAGGAUGACUUGCCGA	21	GGCGAGUUGUUCUUGGCUACA	21	miRCB5174b	Bd3:50778133:50778230	-	CCUCUGUUCCAUAAAGAUUGG	21	CAACCUUUAUGGAACGGAGGG	21
miR169a-p2	Bd2:7704151:7704295	+	UUAGGCUUGGGGACUAUGGUG	21	UCUUAGCUCCUGCCCCUCAUG	21	miRCB5174c-np1	Bd4:40461587:40461716	-	UCCCUCCGUUCUAUGAAGAUUGGC	24	CAAUUUUGCGUGGAACUGAGGGAG	24
miR169b	Bd1:27159089:27159238	-	UAGCCAAGGAUGACUUGCCG	20	GCAGGUUGUUCUUGGCUAACA	21	miRCB5174c-np2	Bd4:40461587:40461716	-	AUGAAGAUUGGCGUGACUUUGAAC	24		
miR169c	Bd5:23763889:23764007	-	CAGCCAAGGAUGACUUGCCGG	21	GGCAGGUUGUCCUUGGCUAC	20	miRCB5174d-np1	Bd1:36001972:36002075	-	UCCCUCCGUUUCAUAAAGAUUGGC	24	CAAUCUUUAUGGAACGGAGAGAGU	24
miR169e	Bd3:43441547:43441670	+	UAGCCAAGGAUGACUUGCCUG	21	GGCAGUCUCCUUGGCUAGC	19	miRCB5174d-np2	Bd1:36001972:36002075	-	UAAAGAUUGGCACGGAUUUGAACU	24		
miR169h	Bd5:11563854:11563977	-	UAGCCAAGGAUGACUUGCCUA	21	GGCAGUCACCUUGGCUAGC	19	miR5178-np1	Bd3:8463684:8463859	-	CUUGGGACCGCCGGUCAGAGC	21	UCUGACCGGUGGGCCUGAGCG	21
miR169j-p1	Bd4:44513774:44513917	+	UAGCCAGGAAUGGCUUGCCUACG	23	UGGUCAAGCCUUCCUGACUAGG	22	miR5178-np2	Bd3:8463684:8463859	-	CGCCGGUCAGAGCCAUGUGGC	21	CAUAUGGCUCUGACCGGUGGG	21
miR169j-p2	Bd4:44513774:44513917	+	CACACAUUUUUCGGUUCAUCACA	23	UGAUGGACGGAGUGUGGCUGC	21	miR5181a	Bd2:48358993:48359091	+	UGAUCCAUAAUAAGUGUCAGG	21	CGGCACUUAUUAUGGAUCAGA	21
miR169k	Bd1:1175446:1175580	+	UAGCCAAGGAUGAUUUGCCUGU	22	GGGCAAGUCAGCCUGGCUACC	21	miRCB5181c	Bd2:22827665:22827764	-	CCUCCGGUCCACAAUAAGUGU	21	ACUUCUUAUGGAUUGUAGGGA	21
miR171c	Bd5:24711130:24711235	-	CGGUAUUGGUGCGGUUCAAUC	21	UUGAGCCGUGCCAAUAUCACG	21	miR5185a	Bd2:41735730:41735830	+	UUCUAGUUCAUUUUUCAAAUC	21	UUUGAGAAUUGAACUAGAAGC	21
miR171d	Bd1:72765327:72765446	-	UGUUGGCUCGACUCACUCAGA	21	UGAUUGAGCCGUGCCAAUAUC	21	miR5185b	Bd5:6094962:6095079	+	UUCUAGUUCAUUUUUCAAAUC	21	UUUGAGAAUUGAACUAGAAGC	21
miR172a	Bd3:55737324:55737435	-	GCAGCACCACCAAGAUUCACA	21	AGAAUCUUGAUGAUGCUGCAU	21	miRCB5185c	Bd1:47573609:47573718	+	UUCUAGUUCAUUUUUCAAAUC	21	UUUGAGAAUUGAACUAGAAGC	21
miR319b-p1	Bd2:45992391:45992582	-	AGAGCUCUCUUCAGUCCACUC	21	UUGGACUGAAGGGUGCUCCCU	21	miRCB5185d	Bd1:66835948:66836081	+	UUCUAGUUCAUUUUUCAAAUC	21	UUUGAGAAUUGAACUAGAAGC	21
miR319b-p2	Bd2:45992391:45992582	-	AGCUGCCGAUUCAUCCAUUCA	21	ACUGGAUGGCACGGGAGCUAC	21	miRCB5185e	Bd1:50946307:50946421	-	UUCUAGUUCAUUUUUCAAAUC	21	UUUGAGAAUUGAACUAGAAGC	21
miR319b-p3	Bd2:45992391:45992582	-	UCAGAUGACUGUAGGGUUUUAUU	23	AAAAAGCUUUUCGUUGUUUGUGGU	24	miRCB5185f	Bd3:44959226:44959335	-	UUCUAGUUCAUUUUUCAAAUC	21	UUUGAGAAUUGAACUAGAAGC	21
miR390	Bd1:2722085:2722258	+	AAGCUCAGGAGGGAUAGCGCC	21	CGCUAUCUAUCCUGAGCUCCA	21	miRCB5185g	Bd4:10669865:10669986	+	UUCUAGUUCAUUUUUCAAAUC	21	UUUGAGAAUUGAACUAGAAGC	21
miR393b	Bd5:27613837:27613967	+	UCCAAAGGGAUCGCAUUGAUC	21	UCAGUGCAAUCCCUUUGGAAU	21	miRCB5185h	Bd4:28275129:28275253	+	UUCUAGUUCAUUUUUCAAAUC	21	UUUGAGAAUUGAACUAGAAGC	21
miR395b	Bd1:55440578:55440668	-	GUUCCCUGCAAGCACUUCACG	21	UGAAGUGUUUGGGGGAACUC	20	miRCB5185i	Bd4:35293520:35293620	+	UUCUAGUUCAUUUUUCAAAUC	21	UUUGAGAAUUGAACUAGAAGC	21
miR395c	Bd5:25456013:25456103	+	GUUCCCUGCAAGCACUUCAUG	21	UGAAGUGUUUGGGGGAACUC	20	miRCB5185j	Bd5:9614368:9614468	-	UUCUAGUUCAUUUUUCAAAUC	21	UUUGAGAAUUGAACUAGAAGC	21
miR395d	Bd3:14764314:14764425	-	UGGGGUUCCCUCCAAACACUUCA	23	AAGUGUUUGGGGAACUCUAGG	21	miRCB5185k	Bd5:24868302:24868411	-	UUCUAGUUCAUUUUUCAAAUC	21	UUUGAGAAUUGAACUAGAAGC	21
miR395e	Bd5:25455202:25455348	+	GUUCUCCUCAAAUCACUUCAGU	22	UGAAGUGUUUGGGGGAACUC	20	miRCB5185l	Bd1:50986582:50986685	-	UUCUAAGUCAAUCUCUAAAUC	21	UUUGGAGAUUGACUUAGAAGC	21
miR395f	Bd5:25455432:25455520	+	GUUCCCUUCAAACACUUUACG	21	UGAAGUGUUUGGGGGAACUC	20	miRCB5185m	Bd5:23787287:23787393	+	UUCUAGUUCAUUUUUCGAAUC	21	UUUGAAAAUUGAACUAGAAGC	21
miR395g	Bd5:25455726:25455812	+	GUUUCCUGCAAACACUUCACG	21	UGAAGUGUUUGGGGGAACUC	20	miR5198	Bd1:3141610:3141866	+	ACAUGGCAGAUAAUCCUUUGAAUC	24	AUUCCAAGGGUUAUCUGACAUGUG	24
miR395h	Bd5:25455875:25455965	+	GUUCCCUGCAAGCACUUCACG	21	UGAAGUGUUUGGGGGAACUC	20	miR5201	Bd2:42461421:42461525	-	UGAUCAUUUGCCCCGUCUUGU	21	CAGGGCGAGGCAAAUGAUCAA	21

Table 2: *Brachypodium distachyon* novel microRNAs.

miRNA name	Genomic localization	S	miRNA 5p	nt	miRNA 3p	nt	miRNA name	Genomic localization	S	miRNA 5p	nt	miRNA 3p	nt
miRCB6	Bd2:4505087:4505190	+	AAUCUUGAUGUGCGGGGAUAG	21	AUUCCCGUACGUCAAGAUUGC	21	miRCB88	Bd3:30460954:30461083	+	GCGGCAUGGUAUUAGAGCAAGUUG	24	ACCUGCUCUGAUACCAUGUUGUGA	24
miRCB8	Bd3:38334085:38334209	+	ACCGGUUUAUUCUGAAGCACCAGU	24	CUAGUGCUUAGACAAAACCCGGUU	24	miRCB89	Bd2:14480283:14480472	-	GCGUCUACUUGGUAUCCAAGCUUA	24	AGCUCUGAUACCAUGUGGAUGAGA	24
miRCB12	Bd4:34312043:34312234	-	ACGGGAUUUUAUAACGGACUUUGG	24	AAAGUUUGGCAUAGAAUUCAAUGC	24	miRCB93	Bd2:50679595:50679690	+	GCUUUCGUAGCUCAGUUGGUU	21	UUCAACUCUCAACGAAAGCAG	21
miRCB13-p1	Bd1:9582818:9582962	+	ACUAGGGAGAUGGUUUUCGCU	21	UGAAAACCAUAUUCCUAGCUC	21	miRCB96a	Bd4:4781925:4782047	+	GUACAUGAACUUAUAAGACGAGUA	24	CAUAGACUUAUGAGACGAGUACAU	24
miRCB13-p2	Bd1:9582818:9582962	+	AUGCUCCACCUCAUAUUUGAC	21	CAAAUAAGAGGAGACGAGACAUAG	24	miRCB96b	Bd1:8415427:8415549	+	GTACATGAACTTATAAGACGAGTA	24	CATAGACTTATGAGACGAGTACAT	24
miRCB14	Bd2:50019627:50020106	-	AGAACAGCCACGGUUGAAAGUUAU	24	AACUUUCUCCCGCAGCUGUUCUGU	24	miRCB97	Bd3:29739922:29740001	-	GUAGAAUGGGUGAUGGGAGAAGGC	24	UUUCUUCUGUGAGCCUGACUGAGC	24
miRCB16	Bd3:47406354:47406435	-	AGAGUCGUUUGUCAAGCUCGG	21	GUGCUUGACAGACGACUCUGG	21	miRCB98	Bd3:42351455:42351563	-	GUCUAUGUCUAUGUCUGUGCACGC	24	UGCACAAACUGUGGAGUAGUCGGC	24
miRCB17	Bd1:41016724:41016952	+	AGAUGCGUCGAAGCGGACUGGAUC	24	UUGAGUCUGAGAAGUAUUUCUG	22	miRCB99	Bd3:39152783:39152894	+	GUGCUCGUCCACAAGAGAAGCAGG	24	UGCUUCGGUCUGUGCUUGGGCACG	24
miRCB19	Bd5:17786144:17786281	+	AGCAUCUACUGAUAGUUUGACUGC	24	UUUGAACUUUUGUAUUGGAUCUUU	24	miRCB100	Bd4:44574033:44574313	+	GUUCAGCCAUACAUCGAUCGAAGC	24	UUUGAUCGAUGUAUGGCUGAACGG	24
miRCB20	Bd1:65104738:65105027	-	AGGAUGGCAAGAUUUAUCGAUGGG	24	CAUCGACAACUUUUGCCAUCCUUA	24	miRCB101	Bd4:31535894:31536302	+	GUUUUUCCUCAGUACCGUUACAAU	24	UGUAAUGGUACUGAACAAAGACGC	24
miRCB21	Bd4:36289096:36289181	-	AGUAAGGCUUUAGUAACAAGAGAU	24	UUGUUGUUACUUAAGUCUUGGUAC	24	miRCB102	Bd3:18724294:18724682	-	UAAAGUAUGACUAGGCACACG	21	UGUGCCUAGUCGUACUUUGAU	21
miRCB25-np1	Bd2:53185475:53185979	-	AUAGACUAAGAGUUCCAACAGAAG	24	UUCUAUUGGAACUCUUAAUCUAUG	24	miRCB103	Bd4:46018159:46018364	+	UACUACAGAUCGUGAUGUCAACUU	24	AUUGAUGUCACAAACUAUAGUAGC	24
miRCB25-np2	Bd2:53185475:53185979	-	UAGACUAAGAGUUCCAACAGAAGC	24			miRCB105-p1	Bd3:4809448:4809995	-	UACUUAGCCUCUUUGACAAUCUUG	24	AGAUUGUUAAAGAGGCCAAGUAUU	24
miRCB26	Bd1:53276108:53276440	-	AUCAGAUUGUUUUGUUGUAGGAUG	24	AUCCUAUAACAAAACAAACUGAUC	24	miRCB105-p2	Bd3:4809448:4809995	-	AUGAUAGAAUUUCUAAAUUAGAAG	24		
miRCB27	Bd1:51138442:51138688	+	AUCCAACAACAAGGGACGUGUUAG	24	AAUAUGUUUUCUAUUGUUGGACGG	24	miRCB105-p3	Bd3:4809448:4809995	-	CCAUAAGUGAAAUCAACUCAUUCU	24	UAUGAGUCGAUUUCUCUUAUGGCU	24
miRCB28	Bd5:16482116:16482261	-	AUCGUCGAGGGCGGAGAUUGCGGC	24	UGGAAUGGGCGCUCUCAGGGAAGC	24	miRCB105-p4	Bd3:4809448:4809995	-	UCUGAAUUUUAUGACCAAGAUAAC	24	UAUCCUAGCCAUAAAAUUCAGUAU	24
miRCB29	Bd4:5126959:5127388	+	AUCUGAAUCAUUGCCGACCAUGCA	24	CAUGGUCGGCAAUGAUACAGACGA	24	miRCB106	Bd4:33900876:33901442	-	UAGAGCUCUGAAGUUACCACCCAC	24	UUAUUGUGGUAACUUUAAGAUGGC	24
miRCB30	Bd3:8931946:8932027	+	AUCUUCCUCGUGGACAAGCGGUAG	24	UUUGGUGCACCCGGCUGGAGAUGG	24	miRCB107	Bd1:72881362:72881695	-	UAGGAAUUGAUGGAACAGCUCACA	24	UUGAGACUGGCAGCAUUAGCAAGC	24
miRCB31	Bd3:3518192:3518609	-	AUGCUCUUCCCACUGUCAUUUUCC	24	AAAAUGAUAGGUUGGAAGAACACGC	25	miRCB109	Bd1:21292473:21292559	-	UAUUUUCUCGGAUCAAUAUUACUU	24	CUAAUAUGUAUCGAAGGGAGUAGC	24
miRCB33	Bd1:22204188:22204374	+	AUGUCUUCUUCCUUGCUCAUC	21	UGAGCAAGGGAGAAGACAUGG	21	miRCB110	Bd3:36497308:36497435	+	UCAAAGAUGGGAUUUCUGAAC	21	UAAGAAAACCCACCUUUGAUG	21
miRCB34	Bd3:16741357:16741575	-	AUGUUAGGAUUCGGUUGACCAAGC	24	UUGGUCAAAGAAAAUCUAAUACGC	24	miRCB111	Bd2:7864984:7865341	-	UCAGUUGCUAGAGAAGAACGAAAC	24	UUCGUUCUUCUCCAGUAAUUGACC	24
miRCB35	Bd3:22735492:22735614	-	AUGUUCUCUCGGCUGAGGAAC	21	UUCUCUCGGCUAAGGAACUGC	21	miRCB112	Bd1:28244716:28244829	-	UCAUUGAGAUUCGUGUAAAUUAA	23	GAUGGAUACGAUUGUCGACUGAGA	24
miRCB37	Bd2:22520134:22520346	-	AUUCCACACUGUAAAUUGAAU	21	UCAAUUUACGGUGUAGAAUUG	21	miRCB113	Bd1:40295515:40296069	+	UCAUUUAUUCGUCCAUGGCGAUGG	24	AUCGCCUUAGACGAAUAAAUGAGG	24
miRCB42	Bd1:55109092:55109290	-	AUUUUGAUACUACUACCUUCAUCA	24	AUGAAGGUAGUAGUUUCAAAAUGG	24	miRCB117	Bd1:40655242:40655380	-	UCCGCCAUACAUGUUUCUAUC	21	UAGAAGCAUAUAUGGCGAAGA	21
miRCB46	Bd1:54324585:54324677	-	CAAUGCAAUGCCAAGUCUUUCGA	23	UGGAGAUGUUGGCAUUGAAUUGGC	24	miRCB118	Bd1:9873496:9873584	-	UCCUUCCGACCCAUAUUACUUGU	23	CAAGUAAUAUGGAUCGGAGGAAGU	24
miRCB47-p1	Bd2:57745334:57745931	+	CACAAAACCUUCAGCUACCCA	21	GGUAGUUGAAUGUUUUGUUUA	21	miRCB119	Bd5:17999321:17999432	-	UCGAAAUUGAUCGUGCGGAGAAGC	24	UUUUACACAUGAUUUGGGUUGGAC	24

miRNA name	Genomic localization	S	miRNA 5p	nt	miRNA 3p	nt	miRNA name	Genomic localization	S	miRNA 5p	nt	miRNA 3p	nt
miRCB47-p2	Bd2:57745334:57745931	+	CUUCCAUAUCAAAUCAUCUCU	21	AGAUAACUUGAUAUGUAAGUG	21	miRCB122	Bd3:35252490:35252674	+	UCUAAGACGACUGAGAAAUAACUA	24	AACUAUUUUUAAGUUGACCGAGAA	24
miRCB51	Bd1:19580903:19581367	+	CACUACCGUUAGUUGACCGUUAAG	24	UAGCGGUCAACUAACUGUAGUGGC	24	miRCB125	Bd1:52273428:52273534	-	UCUCCUCCCGGUACCCUUCUU	21	GAAGGGUAUCGGGAUGAGAGG	21
miRCB54	Bd2:47694041:47694348	+	CAGCCACGUCGGCAUAAGCGAG	22	GGCUUAUGCCGACGUGGCUAC	21	miRCB126	Bd1:55108461:55108695	-	UGAAACAACUAUCUUGGCCUUCUC	24	GAGGGCCAAGGUAGUUGUUUCACA	24
miRCB55	Bd3:44260861:44261035	+	CAGCGGACAGAAUGGAGCAAGCAG	24	GGCUUUGCUCGGAGUGCUGUGGC	23	miRCB127b	Bd1:56080928:56081097	-	UGAACAUGUACAUGCUGGCCAACC	24	UUGGCCCACAUUGACAUGUUCACC	24
miRCB57	Bd3:4507481:4507863	-	CAGUGCCGUCUCUUCCUCAAGUUC	24	UCUUGAUCAAGAGACGGCUCUGGC	24	miRCB127a	Bd1:56107496:56107662	-	UGAACAUGUACAUGCUGGCCAACC	24	UUGGCCCACAUUGACAUGUUCACC	24
miRCB58	Bd5:21791921:21792207	-	CAGUUGAUGACCAAGUUCUCGAGA	24	CUCGGAAUUUGGUCAUCAACUGGC	24	miRCB129-np1	Bd1:1326421:1326660	-	UGACGAGAUCACAUCGUUUGCACA	24	UGCAAACAAUGUGAUUUCGUCAAG	24
miRCB60	Bd4:5125732:5125842	+	CAUAACCUAGAUCUGUAUCA	20	AAAUAGAUCUUGGCGUUAUGGG	22	miRCB129-np2	Bd1:1326421:1326660	-			GCAAACAAUGUGAUUUCGUCAAGA	24
miRCB62	Bd1:49383462:49383925	-	CAUUACAAUUCUUGGGACAUUUGC	24	AAAUGGCCCGAGAAUUGUAAUGGU	24	miRCB130	Bd3:15036602:15036699	-	UGAUGAUGGUACGGACGUCGCGGU	24	UGCGAAUCUGUCACCGUUGUCCCC	24
miRCB63	Bd2:47611750:47612266	+	CAUUUUAGUCCAACAAGUUGCAA	23	UUACAAGAAGUUGGACUAAAAUGC	24	miRCB131	Bd5:13610099:13610196	+	UGAUUAGUUUCAGUUGGUCUGGGC	24	CCAAGUCGAUUGGAACUAAUGAGC	24
miRCB64	Bd1:70862451:70862598	-	CCAACUGGGCCAGUCGGCCUGGGC	24	CGAGGCUGACUGGGACUAAGCGGC	24	miRCB135	Bd2:9800174:9800278	+	UGCUCGGAUUGAGUGUAUUUU	21	AAAUACACUCAAUUCAAGCAG	21
miRCB65-p2	Bd4:9834796:9834906	+	ACGACAGCUUGGACAGGAGGC	21			miRCB137a	Bd3:26812498:26812587	+	UGUUUUCAUAGGCCAUGUAGAGC	23	AGCAAUGGUGGUGGUUUGGAGGAG	24
miRCB65-p1	Bd4:9834796:9834906	+	CCCCAAGCUGAGAGCUCUCCC	21	AGGAGCAAGCAGCUUGAAGGU	21	miRCB137b	Bd2:5081776:5081865	-	UGUUUUCAUAGGCCAUGUAGAGC	23	AGCAAUGGUGGUGGUUUGGAGGAG	24
miRCB66a	Bd5:1447437:1447542	-	CCCGGUCGAGGACGGCCCCGC	21	CGGCGCCGUCCUCGACCGGGAG	22	miRCB141	Bd4:4382662:4383014	+	UUCCAAAUUCCUGAGCAAACAUAU	24	AGGUUUGCUCUGGACUUUGGAAUC	24
miRCB66b	Bd5:21264994:21265108	-	CCCGGUCGAGGACGGCCCCGC	21	CGGCGCCGUCCUCGACCGGGAG	22	miRCB142	Bd3:6980920:6981024	+	UUCCUCCAAGAUCUCGGAGACUC	23	GAUCGAGAUCGUGGAGGAACC	21
miRCB68	Bd1:2375800:2376168	+	CCGUCAGUGCCAACAUGCCAG	21	UGUCAUGUUGGCACUGAUGGG	21	miRCB146-np1	Bd1:38847604:38848027	+	UUCUCGCCAUUGCCAAACCAGGGC	24	CCUGCGUUGGCGAAGGCGAGAAGC	24
miRCB71	Bd2:7993933:7994056	+	CCUUGAAAGCCUGUCUAGACA	21	UCUAGACGGGCCUUCAAAGAG	21	miRCB146-np2	Bd1:38847604:38848027	+	AACGGACGUCUAACUCACUGCAGC	24		
miRCB72	Bd1:65106389:65106525	-	CCUUGACUCCUAUAGUACACAUUC	24	AUGUGUACUAUAGAAGUCAAGGGU	24	miRCB147	Bd1:46850031:46850373	-	UUGAACGUGACUGGGUUAACG	21	UUAACCUAGUCACAUUCAACG	21
miRCB74	Bd2:27051633:27051729	-	CGCUUUUCGGUCCGUGGAGACGCA	24	AGAUCUCCAUGAACUGAGAAACGG	24	miRCB149	Bd3:32702277:32702466	+	UUGCUAUUUCUUGGGCGACUGAGA	24	UUAGUUGACUGAGAAAUAGACGGU	24
miRCB75	Bd1:64869277:64869369	-	CGGGUCAACGAAGGAAAAAUU	21	UUUUUCCUUCGGCUGACACGU	21	miRCB151	Bd1:48106343:48106504	+	UUGUUUUCCUUCUGCACUCCCGGC	24	CCGGUCGGAGCCAAGAGACGCGGC	24
miRCB77	Bd1:72902118:72902245	+	ACUAUGGUCUGUGAGGAUGGCAAC	24	GAGCGAACGUUAAAUUCCGUGGGG	24	miRCB152	Bd4:22303221:22303379	-	UUUACUAUCUGGGAUGUCACA	21	UGACAUAUCUGAUGGUAAAGG	21
miRCB80-p2	Bd2:54086906:54087052	-	ACAUUCAACUCAUUAUUAAUG	21	UUGAUAAUGGGUUGAAUGCGC	21	miRCB154	Bd3:58512458:58512747	-	UUUCGGUCAAUGUAUUUCAAGCAG	24	ACUUGAGACGGACUGUAUUAAAAA	24
miRCB80-p1	Bd2:54086906:54087052	-	CUUGCCCUCUGAUAUCUUGG	20	AAAGAUAUCAGAGGGCAACG	20	miRCB180	Bd3:3759281:3759403	+	UUUGAACAACCUCGGUCUCAU	21	GAGACAGAGGUUGUUCGGAUG	21
miRCB82-p1	Bd1:65552800:65552950	-	CUUUGGUUGGGUAGAACUACC	21	UUGUUCCACCCAACAGAAGAU	21	miRCB181-np1	Bd1:60124988:60125147	-	UUUGAUGACAUGACGUACGGCGGC	24	UGCGAUACGUCACUUCACCGAAAU	24
miRCB82-p2	Bd1:65552800:65552950	-	UCUGACAAACACCAUCGCAAC	21	UGAGAUGGUGUCUGUUGAAGG	21	miRCB181-np2	Bd1:60124988:60125147	-	AUAUUGACUGUAAUUUUGAUGACA	24		
miRCB84	Bd1:18369831:18369977	+	GAGCAUCGUGUCGGCGUGCGCGGC	24	CUGCGCCGCGACUUUCGGACGAG	23	miRCB183-np1	Bd1:39792972:39793171	+	UUUUAAUUGUGGAAGCUCUUG	21	AGAUCUUCCAUGAGUAAAAAU	21
miRCB85	Bd1:38848227:38848545	+	GAGGUCAUUGUCAGACAUGGGAAG	24	UUCUCGAUCUGUAGACCGAUCUAG	24	miRCB183-np2	Bd1:39792972:39793171	+	UCUUGAAGUUUCGCAUGCAGU	21	UGCAUGUGGAACUUCCAGAUC	21
miRCB86	Bd1:68651831:68651930	-	GAUAAUUUAGUGGCCUUCCUACUU	24	GUAGGGACCUUGCUGAAGACGUUU	24	miRCB185	Bd4:31253366:31253495	-	UCAUUCGCUCAUUCACACAGU	21	UGUGUGUUCGAGUGAAUGAGU	21

miRNA name	Genomic localization	S	miRNA 5p	nt	miRNA 3p	nt	miRNA name	Genomic localization	S	miRNA 5p	nt	miRNA 3p	nt
miRCB87	Bd3:6987696:6987971	+	GAUUUUUCUGACGACGGACAUGGC	24	CAUGUCUGUAGUCAGAAAAAUCAA	24							

Table 3: *Brachypodium distachyon* si-RNA like microRNAs

miRNA name	Genomic localization	S	miRNA 5p	nt	miRNA 3p	nt	miRNA name	Genomic localization	S	miRNA 5p	nt	miRNA 3p	nt
miRCB1	Bd1:10809636:10809714	-	AAAGAUUGGCACGGAUUUGAAC	22	UCAAAUCCAUGCCAAUCUUUA	21	miRCB90h-np1	Bd1:15409121:15409326	-	UGCUAUUUCUUAGACGACUGAGA	23	CUUAGACGACUGAGAAAUAGACAC	24
miRCB2	Bd2:18669634:18669888	-	AAAGCAUUCUCGAUGGCCCUGUUG	24	AACAGAUCGUGCGGCGAAUGUGGC	24	miRCB90h-np2	Bd1:15409121:15409326	-	UUAGACGACUGAGAAAUAACUAUU	24	AUAACUAUUCUUAGACGACUGAGA	24
miRCB3	Bd4:31316594:31316675	-	AAAUAGUCGAUCUGUCCGCAGCAG	24	AGAAGUGAGACUCUGAUUGUCAGU	24	miRCB90c	Bd1:26994439:26994640	+	GCUAUUUCUUAGGCGACCGAGAA	23	CUUAAACGACUGAGAAAUAGACAC	24
miRCB4	Bd3:32835972:32836051	+	AAGACAUGUGUUGGUCUGUGGGGC	24	CCUAUAGAGCUGUCAGGAGUGAAG	24	miRCB90g	Bd1:57249729:57249931	+	UUUGCUAUUUCUUAUGCGACUGAG	24	UUAGGCGACCGAGAAAUAGACACU	24
miRCB5	Bd2:22721496:22721638	+	AAGUUAAGAAUUUAGAAUAAGAGG	24	CUCCAUUUCUAAAUACUUGUCGCU	24	miRCB92	Bd4:21298742:21298925	-	GCUAUUUCUUAGGCGACUGAGA	22	CUUAGGCGACUGAGAAAUAGACAC	24
miRCB9	Bd4:42007256:42007334	+	ACCGGUUUCUGUAUUAAAGAUGGC	24	CCAGACCAGAUACAGAGACCGGUC	24	miRCB94	Bd1:54728637:54729025	+	GGAGAGAUGGCUGAGUGGUUG	21	GCAGCUCGGGCUCUUCCUGC	20
miRCB10-np1	Bd2:35086238:35086852	-	ACCUGCUUUUGAUCUCGGUGGCU	23	ACACCAAGAUCGAAAGCAGGGGA	23	miRCB95	Bd3:13969222:13969503	-	GGUGGUGUGGACUCUGGAGUAUGG	24	CUACUCCAAAGAACCACCACUUU	23
miRCB10-np2	Bd2:35086238:35086852	-	UUGGCGGAUCAAGCUCUAACC	21	GUAGAACUUGAUGCGCCAUCGC	22	miRCB108	Bd4:9713704:9713859	-	UAUUUCUUAGUCGAUGAUAACAAC	24	UACGAUUGUCGACUGAGAAAUAAC	24
miRCB11	Bd3:19915230:19915343	-	ACGGCUCUGAUACGUCAACUGAGA	24	UAAGUUGACGAAUCAGAACCGUU	23	miRCB114e	Bd3:38290436:38290536	-	UCCCUCCGUUUCAUAAAGAUUGGC	24	CCGGCCUUUAUGAAACGGAGGGAG	24
miRCB15	Bd3:52452178:52452319	-	AGAAGUUAUGGUCUGUUUGGAUGG	24	AUUUGGCAAGUCAACACUUCAUUG	24	miRCB114d	Bd2:11096916:11097034	+	UCCCUCCGUUUCAUAAAGAUUGGC	24	CAAUCUUUAUGGAACGGAGCGAGU	24
miRCB18	Bd2:6939609:6939695	-	AGAUUGGUGCAUCUUUGAAACGGA	24	UAGUUUAAAACCGUGCCAAUCUUU	24	miRCB114b	Bd2:43485185:43485291	-	UCCCUCCGUUUCAUAAAGAUUGGC	24	CAAUCAUUGUUGAACGGUGGGAGU	24
miRCB22-np1	Bd1:45063148:45063391	+	AGUAUCCGUGCAGUAUCUGAU	21	CGGAUACUGCACGAAUACUGC	21	miRCB114a	Bd1:25194086:25194188	-	UCCCUCCGUUUCAUAAAGAUUGGC	24	CAAUCAUUAUGGCACGGAGGGAGU	24
miRCB22-np2	Bd1:45063148:45063391	+	UGGGUAUGGCAGUGAAAGGAC	21	CCUUUCACUGCCAUACCCAUA	21	miRCB114c	Bd1:52248863:52248969	-	UCCCUCCGUUUCAUAAAGAUUGGC	24	CAAUCUUUAUGAAACGGAGGGAGU	24
miRCB23a	Bd5:22254535:22254622	+	AUAAAGAUUGGCACGGAUUUGAAC	24	CAAAUCCGUGCCAAUCUUUAUGG	23	miRCB115	Bd3:47369841:47369945	-	UCCCUCCGUUUCAUGAAGAUUGGC	24	CAAUCUUUAUGAAACGGAGGGAGU	24
miRCB23b	Bd1:6309876:6309957	+	AUAAAGAUUGGCACGGAUUUGAAC	24	UCAAAUCCAUGCCAAUCUUUAUGA	24	miRCB116	Bd2:3241927:3242029	-	UCCCUCUGUUCCAUAAAGAUUGGC	24	CAAUCUUUAUGGAACGGAGGAAGU	24
miRCB24	Bd3:49409844:49410043	-	AUAAGGGGCUGUUUGGUUUGAACC	24	UUUGAUCCAAACAAGAACCAAAUC	24	miRCB121	Bd3:5897425:5897758	+	UCGGGGUCUGUCGGCAGGAGAGUG	24	AUAGAGACUGCAAUCAGACUGUAG	24
miRCB32	Bd3:7501223:7501611	-	AUGGGGACGGACUGUAAAUUCGUU	24	CGAAAUGCAGUAAUUCUUCUUU	22	miRCB132	Bd1:60236794:60236950	-	UGCACCCUACUUCAUAGCUACAUG	24	GUGUGGCUGUGAAAAGGAAGGCAU	24
miRCB38	Bd2:16835278:16835411	+	AUUGAGAUUCGUGUAAGAUUAAUG	24	AAAAUCUUGAUUGAAUGGCUAUGA	24	miRCB133	Bd3:41704788:41704966	-	UGCGAAAGUCGGCUUCUCGGACAG	24	AGGACCGAGAUUCGAUCAGGCAGC	24
miRCB39	Bd2:4725606:4725737	-	AUUUCUCAGUCGAUGACAACAACU	24	UUACGAUUGUUGACUGAGAAAUA	23	miRCB136	Bd3:22608293:22608402	-	UGUAGUUCAAUUCUCAAAUCU	21	AUUUGAAAAAUGAACUAGAAG	21
miRCB40	Bd2:35842708:35842856	+	AUUUCUUAGUCGAUGAUAACAACC	24	UUACGAUUGUUGACUGAGAAAUA	23	miRCB137c	Bd3:3885672:3885761	+	UGUUUUCAUAGGCCAUGUAGAGC	23	AGCAAUGGUGGUGGUUUGGAGGAG	24
miRCB41	Bd4:33819039:33819193	-	AUUUUAUACGUAUGGGUUUGUAGC	24	UAUGGACACAUACGUAUAAAAUAU	24	miRCB137d	Bd3:9790212:9790301	+	UGUUUUCAUAGGCCAUGUAGAGC	23	AGCAAUGGUGGUGGUUUGGAGGAG	24
miRCB43	Bd3:50819740:50819834	-	CAAGCCGAUUGACUGUAGGCAGGC	24	CCCGCAAGAGGCAGUCGGUCUGGC	24	miRCB139f	Bd3:39049835:39050018	+	UUAGGCGACUGAGAAAUAACUAUU	24	UAACUAUUUCUUAGGCGACUGAGA	24
miRCB44	Bd4:6410674:6410812	+	CAAGGAUUCUCUGCAGUAAGUCAC	24	GACUUAAAGUCGGACAAUCCACGC	24	miRCB139k-np1	Bd3:41195680:41195863	+	UUAGGCGACUGAGAAAUAACUAUU	24	UAACUAUUUCUUAGACGACUGAGA	24
miRCB45	Bd3:49672105:49672188	+	CAAGUUUAGGUGAAGUGAAGUAGG	24	CUGACUCCAGUAAAAUGAACUAGA	24	miRCB139k-np2	Bd3:41195680:41195863	+	GCUAUUUCUUAGGCGACUGAGA	22	CUUAGACGACUGAGAAAUAGACAC	24
miRCB48	Bd1:67036008:67036429	+	CACAGUUGUUGCUAACAGUCUCAU	24	GAGACUGUUAGCAACAACUGUGGU	24	miRCB139j	Bd3:42162488:42162670	-	UUAGGCGACUGAGAAAUAACUAUU	24	UAACUAUUUCUUAGACGACUGAGA	24
miRCB49	Bd3:19729421:19729787	+	CACAUGAUGGAGAGCCAGAGGUGC	24	ACGGAACGGCUCUGCACGGGCGGC	24	miRCB139a	Bd4:23569825:23570006	-	UUAGACGACUGAGAAAUAACUAUU	24	UAACUGUUUCUUAGGCGACUGAG	23

miRNA name	Genomic localization	S	miRNA 5p	nt	miRNA 3p	nt	miRNA name	Genomic localization	S	miRNA 5p	nt	miRNA 3p	nt
miRCB52	Bd5:13714137:13714697	-	CACUACUGUUAGUUGACCGCUAGU	24	UAGCGGUCAACUGACGGUAGUGGC	24	miRCB139g	Bd4:36750958:36751141	-	UUAGGCGACUGAGAAAUAACUAUU	24	UAACUAUUUCUAAGUCAACUGAGA	24
miRCB53	Bd1:28951378:28951824	+	CACUGUUAACCUGACAAGUAGGC	23	CUAGUUGUCAUGUUUUUUGGC	21	miRCB139e	Bd2:4033447:4033630	-	UUAGGCGACUGAGAAAUAACUAUU	24	AUAACUAUUUUUUAGGCGACUGAG	24
miRCB56	Bd1:74158217:74158528	+	CAGUAAGGGUCGGAGAAGG	19	AUCGUUGGCCUCUAUGGUA	19	miRCB139h	Bd3:13865689:13865872	+	UUAGGCGACUGAGAAAUAACUAUU	24	UAACUAUUUCUUAGGCGACUGAGA	24
miRCB59	Bd3:38303678:38303757	+	CAUAAAGAUUGGCACGGAUUUGAA	24	CAAAUUCAUGUCAAUCUUUAUGGA	24	miRCB139d	Bd2:9807658:9807838	+	UUAGGCGACUGAGAAAUAACUAUU	24	AAUAACUAUUUCUUAGGCGACUGA	24
miRCB61	Bd4:334013:334224	+	CAUCAAUUUCCAUGCAUGUUCUG	23	AGAUUAUCGUGCAAGAGACGAGCA	24	miRCB139l	Bd1:9884128:9884312	+	UUUAGGCGACCGAGAAAUAACUAU	24	AACUAUUUCUUAGUCGACUGAGAA	24
miRCB67	Bd5:19365162:19365306	-	GCUUCUAGUUCAUUUUUCAAA	21	UUUGAGAAUUGAACUAGAAGC	21	miRCB139i	Bd3:19994624:19994807	+	UUAGGCGACUGAGAAAUAACUAUU	24	UAACUAUUUCUUAGGCGACUGAGA	24
miRCB73	Bd2:50568774:50568931	-	CGACGGUGUGUUCUGGCAGAGGC	23	AGAAGAUCGGGAGAGGCCGGGUGG	24	miRCB139c	Bd1:49766312:49766489	+	UUAGGCGACUGAGAAAUAACUAUU	24	UAACUAUUUCUUAGGCGACUGAGA	24
miRCB76	Bd2:41411557:41411714	-	CUAGCACAACGCCACAACUGAGAU	24	AUUGAGUUGUAGAUCUGUGAGGAG	24	miRCB139b	Bd1:53151585:53151768	+	UUAGGCGACUGAGAAAUAACUAUU	24	UAACUAUUUCUUAGGCGACUGAGA	24
miRCB78	Bd1:35760796:35761225	+	CUGUAGCUGGAUUCCCCGGAAC	22	UCAAAAGAGGAAAGGCUUGCGGUG	24	miRCB140a	Bd4:32511953:32512217	-	UUAGUCCAUGAACUCAGAAAACGC	24	AUUUUCUAAGUUUGUGGACUAAAG	24
miRCB79	Bd4:3157502:3157710	+	CUUAGACGGCUGAGAAAUAACUAU	24	AACUAUUUUUUAGACGACCGAGAA	24	miRCB140b	Bd4:36681274:36681719	-	UUAGUCCAUGAACUCAGAAAACGC	24	GUUUUCUGAGUUCGUGAACUAAAG	24
miRCB83	Bd4:34950395:34950757	+	GAAGUGUCCAUGAUUGUCAGACGG	24	GUAGAAUGAUUUGUGGGCCUGCAA	24	miRCB144	Bd4:27998773:27998872	+	UUCUAGUUCAAUUCUCAAAUC	21	UUUGAAAAAUGAACUAGAAGC	21
miRCB90i-np1	Bd5:17015404:17015611	+	UUGCUAUUUCUUAGACGACUGAGA	24	CUUAGGCGGCUGAGAAAUAGACAC	24	miRCB150	Bd4:37430830:37430945	+	UUGGUCAAAGAAAAGCUAAUACGC	24	AUAUUAGGUUUCGGUUGACUAAGC	24
miRCB90i-np2	Bd5:17015404:17015611	+	UUAGACGACUGAGAAAUAACUAUU	24	AUAAUUAUUUCUUAGGCGGCUGAG	24	miRCB155b	Bd3:49689637:49689783	-	UUUCUCAAUCGAUAAUAACAACCU	24	GCUACGAUUGUUGACUGAGAAAUA	24
miRCB90e	Bd5:24869810:24869993	+	GCUAUUUCUUAGGCGACUGAGA	22	CUUAGGCGACUGAGAAAUAGACAC	24	miRCB155a	Bd4:42605401:42605547	+	UUUCUCAAUCGAUAAUAACAACCU	24	GCUACGAUUGUUGACUGAGAAAUA	24
miRCB90b	Bd4:3245568:3245808	+	GCUAUUUCUUAGACGACUGAGAA	23	CUUAGGCGACUGAGAAAUAGACAC	24	miRCB184	Bd3:29243710:29243814	-	UUUUGCGACCUAUUCACAUCAGGA	24	UUGGAUUUGAAACUUUGCAAGGUG	24
miRCB90d	Bd4:31621922:31622105	+	GCUAUUUCUUAGGCGACUGAGA	22	CUUAGACGACUGAGAAAUAGACAC	24	miRCB187	Bd3:28216817:28217027	+	UUUGCUAUUUCUCAGGCGACUGAG	24	UAGGCGACUGAGAAAUAGACACUC	24
miRCB90a	Bd2:35726856:35727065	-	GCUAUUUCUUAGACGACUGAGAA	23	CUUAGACGACUGAGAAAUAGACAC	24	miRCB188	Bd3:50672314:50672518	+	UUUGCUAUUUCUUAAACGACUGAG	24	UAGGCGACUGAGAAAUAGACACUC	24
miRCB90f	Bd2:50620353:50620563	-	UCUAUUUCUCAGUCGACUGAGA	22	UUAGGCGACUGAGAAAUAGAC	21							

Table 4: Differentially expressed miRNAs during development under control condition (Proliferating cells vs Expanding cells)

miRNA name	Type	Pc	Ec	Fold Change	log2 Fold Change	FDR	Target
miRCB159b-p2	star	53.93030575	5.130431153	0.095130763	-3.39394424	0.197030563	N.A.
miR160d	star	63.15981791	3.270813619	0.051786305	-4.27128555	0.131812917	Glycine-rich RNA-binding protein*
miRCB166f	star	229.6492755	10.20591919	0.04444133	-4.4919542	0.130682769	Reverse transcriptase*

Table 5: Differentially expressed miRNAs during development under stress condition (Proliferating cells vs Expanding cells).

miRNA name	Type	Ps	Es	Fold Change	log2 FoldChange	FDR	Target
miR156b	mature	10732.48063	72874.14362	6.790055917	2.763423455	0.020977324	SPL
miR156b	star	19.04889088	133.7021294	7.018893133	2.811243538	0.181063143	Transcription elongation factor *
miR156d	mature	10732.48063	72874.14362	6.790055917	2.763423455	0.020977324	SPL
miR156d	star	16.83387423	135.8430236	8.069623293	3.012501327	0.120795326	N.A.
miRCB156e	mature	10732.48063	72874.14362	6.790055917	2.763423455	0.020977324	SPL
miRCB156f	mature	10732.48063	72874.14362	6.790055917	2.763423455	0.020977324	SPL
miRCB156g	mature	10732.48063	72874.14362	6.790055917	2.763423455	0.020977324	SPL
miRCB156h	star	8.542983499	103.0644803	12.06422561	3.592663408	0.060635727	ATP binding / Nutrient reservoir activity
miRCB156h	mature	10732.48063	72874.14362	6.790055917	2.763423455	0.020977324	SPL
miRCB156i	mature	10732.48063	72874.14362	6.790055917	2.763423455	0.020977324	SPL
miR159	mature	256.6312147	1306.795301	5.09211361	2.348264607	0.19192039	MYB65 / MYB33
miR160d	star	43.85098672	3.214063413	0.073295122	-3.770139008	0.117480414	Ca responsive / WAK receptor *
miR164a	mature	529.1125592	13508.28122	25.53007104	4.674125647	4.03E-06	NAM
miR164a	star	11.622936	124.357993	10.69936142	3.419452788	0.06659658	TCP *
miR164c	mature	52.57548662	323.9097809	6.160851791	2.62312983	0.190092828	NAM
miR164c	star	0.355164287	10.68810093	30.0933999	4.911375204	0.128751085	Lipase *
miR166a	star	6.22489533	88.15093521	14.16103092	3.823854392	0.052929682	N.A.
miRCB166f	star	211.5243291	6.774339903	0.032026292	-4.964599422	0.014498503	Reverse transcriptase *
miR167c	mature	8.594519261	1733.851338	201.7391881	7.656347546	1.06E-10	Symplekin *
miR167c	star	6.986759666	593.2731854	84.91392487	6.407929253	2.44E-06	Histone-lysine N-methyltransferase/Acetyltransferase activity *
miR167d	mature	23.29740777	2910.073029	124.9097349	6.964742108	1.44E-10	Simplekin*
miRCB167e	mature	23.29740777	2910.073029	124.9097349	6.964742108	1.44E-10	Simplekin*
miRCB167e	star	115.6926794	4193.6966	36.24859	5.179853	2.23E-06	MYB related TF/ UDP-glucose-6-dh *
miR169a-p2	mature	0.355164287	10.70900163	30.15224788	4.914193655	0.139786654	Serpins *
miR169a-p1	star	0	5.693899272	Inf	Inf	0.183757423	Phosphoglycerate mutase/Glycosyl hydrolase *
miR169b	star	28.88255089	255.9474003	8.861661884	3.147577282	0.060089319	NBS-LRR/Chaperone binding protein *
miR319b	mature	742.8651193	102.3102657	0.137723879	-2.860149376	0.241548174	TCP2
miR395b	mature	253.1960979	1540.141505	6.08280111	2.604735833	0.111294138	APS4/F-box
miR395c	mature	253.1960979	1540.141505	6.08280111	2.604735833	0.111294138	APS4
miR395d	mature	0.355164287	12.28831802	34.59896857	5.112657125	0.105844027	Starch Branching Enzyme *
miR395e	mature	253.1960979	1540.141505	6.08280111	2.604735833	0.111294138	APS4
miR395f	mature	253.1960979	1540.141505	6.08280111	2.604735833	0.111294138	APS4
miR395g	mature	253.1960979	1540.141505	6.08280111	2.604735833	0.111294138	APS4
miR395h	mature	253.1960979	1540.141505	6.08280111	2.604735833	0.111294138	APS/F-box
miR395j	mature	253.1960979	1540.141505	6.08280111	2.604735833	0.111294138	APS4
miR395k	mature	253.1960979	1540.141505	6.08280111	2.604735833	0.111294138	APS4
miR395l	mature	253.1960979	1540.141505	6.08280111	2.604735833	0.111294138	APS4
miR395n	mature	253.1960979	1540.141505	6.08280111	2.604735833	0.111294138	APS4
miRCB395o	mature	253.1960979	1540.141505	6.08280111	2.604735833	0.111294138	APS4
miR396b	mature	700.7394393	14830.21994	21.16367241	4.403518086	1.03E-05	GRF1/GRF2
miR396b	star	9.763083647	132.2609328	13.54704492	3.759906279	0.027051103	AUX-IAA/QLQ *
miR396e	mature	446.8096476	2536.00046	5.675796111	2.504822765	0.091290874	GRF2 & SYP131
miR408	star	0	20.88459756	Inf	Inf	0.006022145	TCP14
miR528	mature	4317.40729	38969.22684	9.026071489	3.174098205	0.009370545	XBAT32

miRNA name	Type	Ps	Es	Fold Change	log2 FoldChange	FDR	Target
miR827	mature	187.8324145	1774.668313	9.448147266	3.240031452	0.016917731	Serine/Threonine protein kinase
miR5168-np1	mature	3431.212689	14605.9974	4.256803273	2.089770418	0.141484103	N.A.
miRCB84	mature	17.085967	133.0987501	7.789945407	2.961613218	0.149232958	N.A.
miRCB100	mature	0.845895258	55.63682953	65.77271715	6.039417366	0.002569587	Tetratricopeptide repeat *
miRCB125	mature	0.710328575	12.39917929	17.45555471	4.125614299	0.164907848	UDP-Glycosylt ransferase

Table 6: Differentially expressed miRNAs depending on environmental condition, in proliferating cells (Stress vs Control).

miRNA name	Type	Pc	Ps	Fold Change	log2 Fold Change	FDR	Target
miR169j	mature	76.74679649	8.898147786	0.115941618	-3.108529567	0.119816986	NF-YA2, 4, 9*
miRCB141	mature	803.3165882	211.2811259	0.263011033	-1.926804773	0.176501827	MULE transposase domain*
miRCB78	mature	93.0801974	10.28630978	0.110510184	-3.177748764	0.086905405	N.A.
miRCB22-np2	star	8.990033454	0.845895258	0.09409256	-3.409775545	0.105521221	NAC domain/NBS-LRR

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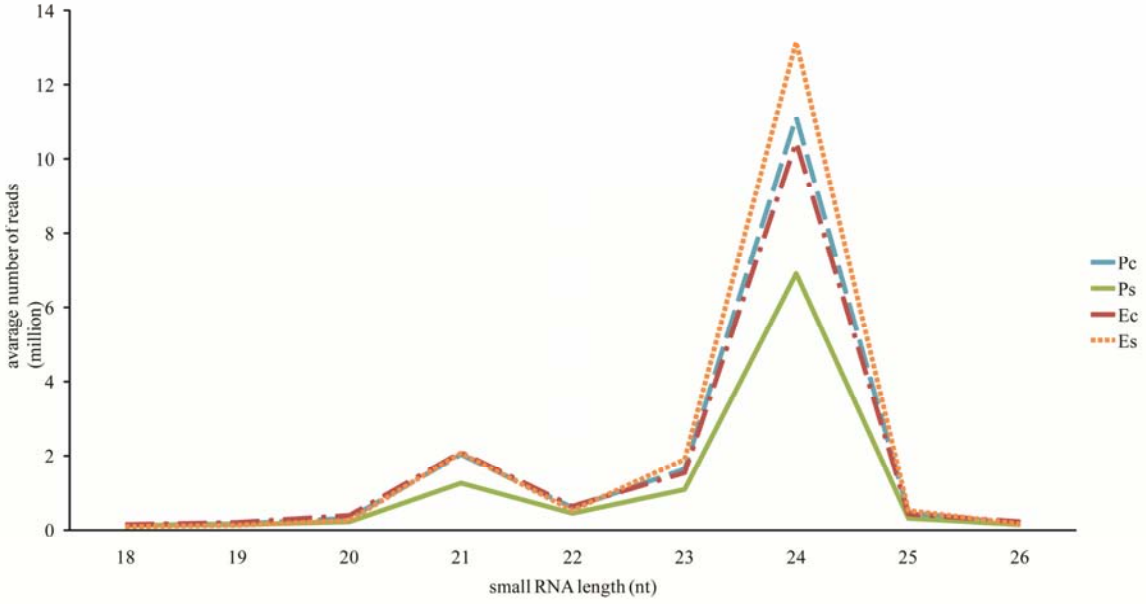
Figure: 1



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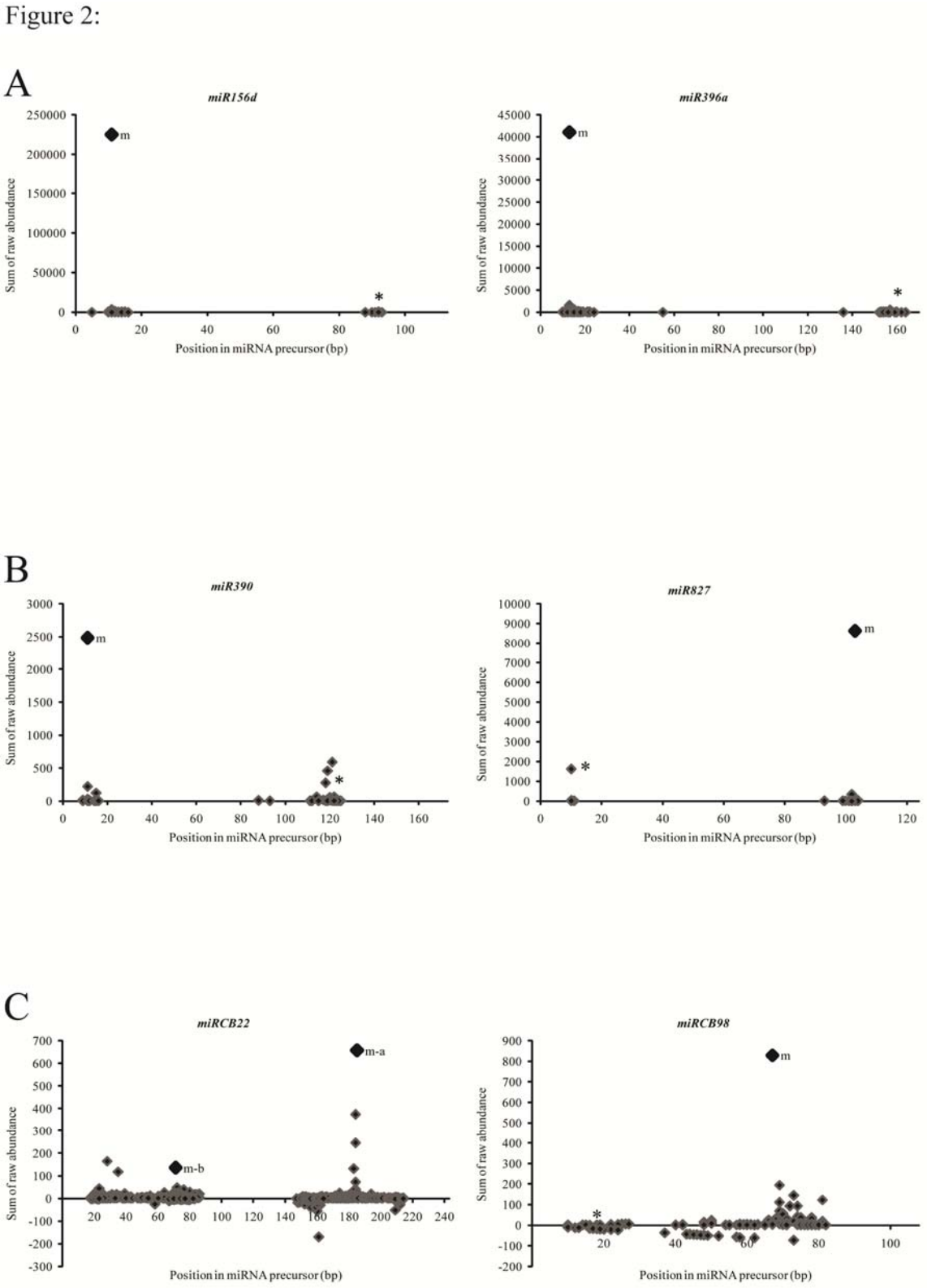
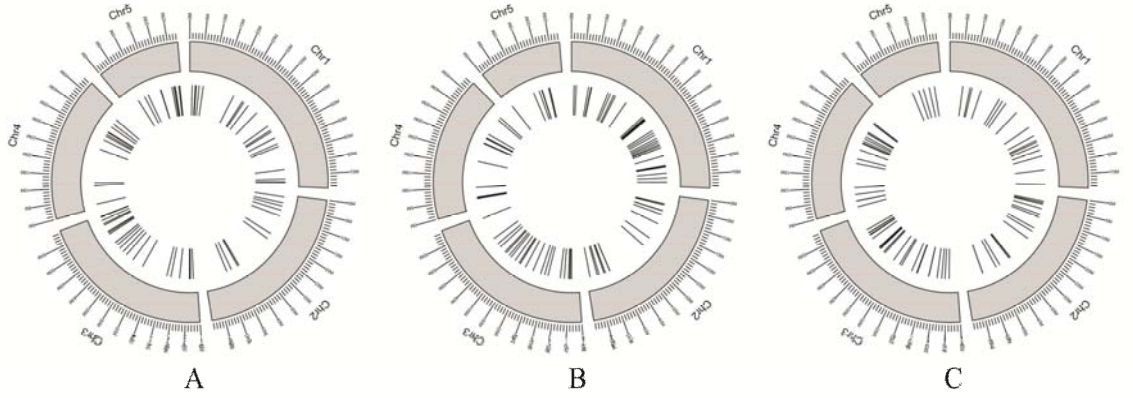


Figure 3:

Figure 4:



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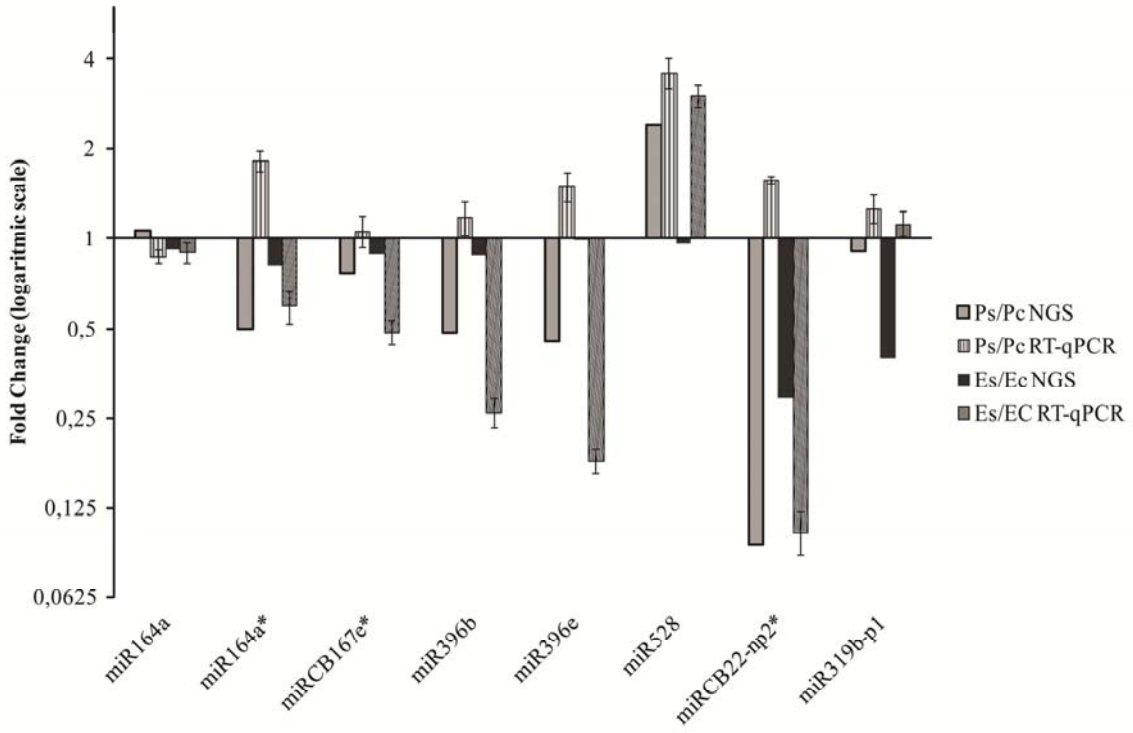

Figure 6