Addressing the Role of microRNAs in Reprogramming Leaf Growth during Drought Stress in *Brachypodium distachyon*

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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 evidence for an 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.

**Key words:** 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; Tester and Langridge, 2010; Godfray et al., 2011). 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 (Cecarelli 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 (Tester and Langridge, 2010; Godfray et al., 2011).

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

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doi:10.1093/mp/sss160, Advance Access publication 21 December 2012

Received 13 November 2012; accepted 15 December 2012
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; Skirycz et al., 2010; Iyer-Pascuzzi et al., 2011; Skirycz et al., 2011). 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 (Veselov et al., 2002; Fricke et al., 2006).

In the past decade, a variety of pathways in which small RNAs (sRNA) guide gene silencing through sequence complementarity-dependent transcriptional and posttranscriptional 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). MicroRNAs 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 sRNAs that resemble miRNAs, but do not conform to these strict criteria, are called sRNA-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 (Lu et al., 2005; Ding and Voinnet, 2007; Chitwood et al., 2009; Rubio-Somoza et al., 2009; Dunoyer et al., 2010; Molnar et al., 2010; Sunkar, 2010; Rubio-Somoza and Weigel, 2011).

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., 2007, 2009) and Oryza sativa, where the involvement of miRNAs in abiotic stress response has also been investigated (Zhao et al., 2007; Shen et al., 2010; 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 (Vogel et al., 2010; Brkijac et al., 2011), 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 in cell expansion (Vereist 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 (Unver and Budak, 2009; Wei et al., 2009; Zhang et al., 2009; Baev et al., 2011) 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 an 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 the Methods section, we set up an in vivo drought assay of Brachypodium plants, shown to cause a dramatic reduction in leaf size mostly affecting cell expansion (Vereist et al., 2012). We collected the third leaf (leaf 3) of Brachypodium plants grown in control and stress conditions (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 eight libraries (Ps1, Ps2, Pc1, Pc2, Es1, Es2, Ec1, Ec2). Each library was subjected to deep sequencing using the Illumina GAIIx 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 Supplemental Table 1 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 (Supplemental Table 1).

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 21-nt molecules but, on average, each unique 21mer shows higher representation in the libraries (see Figure 1 and Supplemental Figure 1) consistently 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, last accessed February 5, 2013) 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: (1) Support Vector Machine output value, (2) strand abundance, (3) strand bias, and (4) duplex bias (see the Methods section for a detailed description of statistical parameters), excluding candidates where two 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 (Unver and Budak, 2009; Wei et al., 2009; Zhang et al., 2009; Baev et al., 2011). Of these, less than one-third (39 of 142) were independently validated. Our predictions included 66 of these loci (see Supplemental Tables 2–4). In addition to those previously deposited MIR genes, 28 new miRNA genes belonging to already-known miRNA gene families were identified (see Supplemental Tables 2 and 3). 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 11 *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 *miR203*, *miR5202*, *miR5200*, *miR5199*, and *miR5176* that have been experimentally validated in other monocot species. However, our pipeline identified 94 previously undiscovered and potentially Brachypodium-specific miRNAs whose precursors show no significant similarity to precursor sequences in miRBase (Supplemental Table 3). Based on the similarity of mature miRNA sequences, potential candidates were grouped into 90 gene families, of which only four have more than one member. For all the predicted fold-back structures presented here, our pipeline identified both miRNA and miRNA* sequences, reinforcing the validity of the predictions, as suggested by Meyers and collaborators (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 (Supplemental Table 4).

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**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.
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 the University of Delaware (http://smallrna.udel.edu, last accessed February 5, 2013). 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 Supplemental Figures 2 and 3). 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 fewer 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 2A and 2B). 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 2C). 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 posttranscriptional 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 (Supplemental Table 3), 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 (miR519B, 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 Supplemental Figure 4A and 4B). 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 11 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 (Mi et al., 2008; Devers et al., 2011; Meng et al., 2011). To increase specificity of predictions, only target predictions provided by both methods were considered (see Supplemental Tables 5–7). 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 nine 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 (Hemler, 2003; Cnops et al., 2006).
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 Supplemental Table 5). Most of such targets are transcription factors, such as SPL, involved in vegetative phase change; AUXIN RESPONSE FACTOR (ARF), involved in organ elongation; HD-ZIPIII genes, such 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, such as 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
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 black bars.
repeat expression, implicating it 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 consistently with accurate dissection of the leaf tissue. To provide an overview of miRNA expression levels, the sum of the abundances of all eight libraries was calculated and expressed as number of tags per five million (TP5M) (Supplemental Tables 2–4).

Almost all highly conserved miRNAs have an overall abundance greater than 30 TP5M (90th percentile is ~16 000 TP5M) with some, such as miRNA156 and miRCB159b, expressed at more than 16 000 TP5M (see Supplemental Table 2). miRCB159b, whose targets include MYB65 and MYB33 transcription factors and potentially Histidinol–phosphate transaminase, was the most highly expressed miRNA, with 228 381 TP5M. Putatively novel miRNAs are represented at relatively lower expression levels, ranging from 1 to 12 000 TP5M (90th percentile corresponds to ~1 100 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, miRCB35, miRCB141)—an indication of a potential biological role for both of these molecules, as suggested previously (Mi et al., 2008; Devers et al., 2011; Meng et al., 2011). 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 versus control in the same developmental area and proliferating versus 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 1, 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 2, 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 cell proliferation cell regulation (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 miR164a, miR164c, miR396b, and miR396e up-regulated in expanding cells (log2-fold change: 2.7, 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 supports 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 versus stress conditions, we identified four miRNAs with significantly changed expression levels (Table 3, 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

| 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 1. Differentially Expressed miRNAs during Development under Control Conditions (Proliferating Cells versus Expanding Cells).

List of microRNAs showing a differential expression depending on developmental area, in control conditions (proliferating cells versus 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 2. Differentially Expressed miRNAs during Development under Stress Conditions (Proliferating Cells versus Expanding Cells).

| miRNA name | Type   | Ps       | Es       | Fold change | log2 fold change | FDR          | Target                                                                 |
|------------|--------|----------|----------|-------------|------------------|--------------|-------------------------------------------------------------------------|
| miR156b    | mature | 10732.48063 | 7287.14362 | 6.790055917 | 2.763423455     | 0.020977324 | SPL                                                                    |
| miR156b    | star   | 19.04898088 | 133.701294 | 7.018893133 | 2.811245358     | 0.181063143 | Transcription elongation factor*                                          |
| miR156d    | mature | 10732.48063 | 7287.14362 | 6.790055917 | 2.763423455     | 0.020977324 | SPL                                                                    |
| miR156d    | star   | 16.83387423 | 135.840236  | 8.069623293  | 3.012501327     | 0.120795326 | N.A.                                                                   |
| miRCB156e  | mature | 10732.48063 | 7287.14362 | 6.790055917 | 2.763423455     | 0.020977324 | SPL                                                                    |
| miRCB156f  | mature | 10732.48063 | 7287.14362 | 6.790055917 | 2.763423455     | 0.020977324 | SPL                                                                    |
| miRCB156g  | mature | 10732.48063 | 7287.14362 | 6.790055917 | 2.763423455     | 0.020977324 | SPL                                                                    |
| miRCB156h  | star   | 8.542983499  | 103.064403  | 12.06422561  | 3.592663408     | 0.060635727 | ATP binding/nutrient reservoir activity                                  |
| miRCB156h  | mature | 10732.48063 | 7287.14362 | 6.790055917 | 2.763423455     | 0.020977324 | SPL                                                                    |
| miRCB156i  | mature | 10732.48063 | 7287.14362 | 6.790055917 | 2.763423455     | 0.020977324 | SPL                                                                    |
| miR159     | mature | 256.6312147  | 1306.795301 | 5.09211361  | 2.348624607     | 0.19192039  | MYB65/MYB33                                                            |
| miR160d    | star   | 43.85098672  | 3.214063413 | 0.073295122 | –3.770139008    | 0.11748014  | Ca responsive/WAK receptor*                                             |
| miR164a    | mature | 529.1125592  | 13508.28122 | 25.53007104 | 4.674125647     | 4.03E-06    | NAM                                                                   |
| miR164a    | star   | 11.622936   | 124.357993  | 10.69936124  | 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 | 14.1610392   | 3.823854392     | 0.052929682 | N.A.                                                                  |
| miRCB166f  | star   | 211.5243291  | 7743.93903  | 0.032026922 | –4.964999422    | 0.014498503 | Reverse transcriptase*                                                  |
| miR167c    | mature | 8.594519261  | 1733.581338 | 201.7391881  | 7.656347546     | 1.06E-10    | Symplekin*                                                             |
| miR167c    | star   | 6.986759666  | 593.2731854 | 84.91392487 | 6.40792953      | 2.44E-06    | Histone-lysine N-methyltransferase/acyltransferase activity*           |
| miR167d    | mature | 23.29740777  | 29010.73029 | 124.9097349  | 6.964742108     | 1.44E-10    | Simplekin*                                                             |
| miRCB167e  | mature | 23.29740777  | 29010.73029 | 124.9097349  | 6.964742108     | 1.44E-10    | Simplekin*                                                             |
| miRCB167e  | star   | 115.6926794  | 4193.6966   | 36.24859    | 5.179853        | 2.23E-06    | MYB-related TF/JDP-glucose-6-dh*                                      |
| miR169a-p2 | mature | 0.355164287 | 10.70900163 | 30.15224788 | 4.911493655     | 0.139786654 | Serpins *                                                              |
| miR169a-p1 | star   | 0          | 5.69389972  | Inf         | Inf             | 0.183757423 | Phosphoglycerate mutase/glycosyl hydrodase*                             |
| miR169b    | star   | 28.88250898  | 255.9474003 | 8.861661884 | 3.147577282     | 0.060089319 | NBS-LR/chaperone binding protein*                                      |
| miR319b    | mature | 742.8651193  | 102.310265  | 0.137723879 | –2.86014376     | 0.021548174 | 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     | 0.10584027  | 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 | APS4/F-box                                                             |
| miRNA name | Type  | Ps       | Es        | Fold change | log2 fold change | FDR         | Target                                      |
|------------|-------|----------|-----------|-------------|-----------------|-------------|---------------------------------------------|
| 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 | 21.16376241 | 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                                      |
| miR827     | mature| 187.8324145  | 1774.686313 | 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    | 13.0987501  | 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-glycosyl transferase                    |

List of microRNAs showing a differential expression depending on developmental area under stress conditions (proliferating cells versus 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.
by drought stress in an 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 overexpressed (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-1p, bdi-miR167c, bdi-miRCB167e star, bdi-miR396b, bdi-miR396e, bdi-miRCB22-np2star, miR169j, miR5185, miR395b, miRCB159b, and miR156b) identified by the NGS approach (see Tables 1–3). 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 eight 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 Supplemental Figure 5). Fold change in miRNA expression in the Pc vs Ps, Ec vs Es, Pc vs Ec, and Ps vs Es comparisons as estimated from RT-qPCR 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, mir164astar, mir167, mir167star, mir396b, mir396e, and mir528 are confirmed as more expressed in the expansion tissue than in proliferation both in control and stress conditions (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 mirRCB22-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 Supplemental Figure 6). 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 four Rice TAS3 loci annotated in the Cereal Small RNA Database (http://crrdb.ucdavis.edu/cgi-bin/smrna_browse/rice2/, last accessed February 5, 2013). 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 posttranscriptional 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 RNA 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 (Unver and Budak, 2009; Wei et al., 2009; Zhang et al., 2009; Baev et al., 2011). 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 into 17 highly conserved, 106 less conserved, or novel miRNA 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 miR399 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; Sunkar et al., 2006; Gu et al., 2010; Zhu et al., 2011). miR162 targets DCL1, involved in miRNA biogenesis, and only four 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 structure 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-nt-long molecules (65% were 23–24 nt long, while 35% were 20–22 nt long). Twenty-four base miRNAs are also common in rice (Wu et al., 2010) where their biogenesis is dependent on DCL3. Interestingly, while rice encodes two DCL3 proteins—DCL3A and DCL3B (Kapoor et al., 2008; Wu et al., 2010)—Brachypodium encodes an additional DCL3A homolog (Supplemental Figure 7). 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 are 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 (Zhao et al., 2011) and recent reports suggest that many hundreds

### Table 3. Differentially Expressed miRNAs Depending on Environmental Conditions in Proliferating Cells (Stress versus 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 | N.A. |
| miRCB141   | mature | 803.3165882 | 211.2811259 | 0.263011033 | -1.926804773 | 0.176501827 | N.A. |
| miRCB87    | 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 | N.A. |

Differentially expressed microRNAs depending on environmental conditions (drought versus control condition) in proliferating cells. Pc, proliferating cells under severe drought stress; Ps, proliferating 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.
Figure 5. Expression Fold Change of Different miRNAs among Two Experimental Conditions. 

(A) Expanding versus proliferating cells in either control or stress conditions. For each miRNA, the fold changes resulting from deep sequencing data (NGS) and from the real-time approach (RT–qPCR) are shown, in order to directly compare the two techniques.

(B) Stress versus control in either proliferating or expanding cells. For each miRNA, the fold changes resulting from deep sequencing data (NGS) and from the 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 Four Identified Brachypodium TAS3 Loci. 
3' and 5' miR390 target sites are shown. Shaded boxes represent the most highly conserved regions corresponding to the expected mature ta-siRNAs from rice and Arabidopsis.
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 by 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 (Kidner, 2010; Johnson and Lenhard, 2011). 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 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 during the early phases of leaf development and final leaf size (Kidner, 2010; Martin-Trillo and Cubas, 2010). 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. Consistently 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 (Vroemen et al., 2003; Koyama et al., 2010; Hasson et al., 2011). 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, and 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 an 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; Sobeh 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 a 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-Per al 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 >222 000 TPSM) 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 overexpression 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 overexpressing 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 posttranscriptional 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. Overexpression of snAC1 enhances drought tolerance through 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 h 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 by 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 h of light, at 24°C and 55% relative humidity. After 3 days, when all seeds had germinated synchronously, individual plantlets, comprising only first leaf, were carefully transferred to pots (5.2 cm in 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-h time window.

Construction and Sequencing of Small RNA Libraries

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

Eight small RNA libraries (four tissues and two biological replicates) were prepared using a 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 an Illumina Genome Analyzer Ix. Sequencing was performed at Applied Genomics Institute (IGA), Udine, Italy.

Our raw data have been submitted to SRA Archive with the following i.d.: 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, 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 four 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 five 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 10 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 six 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 two 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, 2011) 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 is 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 is the proportion of all reads mapping to the candidate precursor region, on the expected strand, that are involved in canonical miRNA:miRNA* interactions (two base 3’ overhangs). For cleanly processed miRNA precursors, this value is expected to approach 1. Strand bias is 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 these statistics and then manually examined. We considered putative miRNAs all the structures whose mean value comprises 1 and 0.65. Sequence similarity searches were performed against miRBase to identify homologs of known miRNA families from Brachypodium and/or other plant species.

The linear count scaling method TP5M was chosen for data comparison according to the following formula: normalized abundance (TPM) = raw abundance/(total genome match – t/r/s/n/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 phytozone.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 the 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 by Sato et al. (2012); briefly, a maximum of three 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). The sum of abundances of all the variants identified per each miRNA candidate was reported for each small RNA library (see Supplemental Figure 2).

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 two candidate miRNAs: psRNATarget (www.plantgrn.org/psRNATarget), last accessed February 5, 2013 (Dai and Zhao, 2011) and TARGET FINDER release 1.6 (www.carringtonlab.org/resources/targetfinder, last accessed February 5, 2013) (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 cut-off: 2.5 for psRNA Target (where the default parameter is set to 3) and 4 (the suggested score cut-off) for TARGET FINDER, selecting only those targets that were identified by both algorithms, in order to reduce false positive rates.

Differential Expression Analysis of miRNAs under Drought Stress

Differential expression analyses 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 57 500 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 cut-off ≤0.2 were applied. Differentially expressed miRNAs during drought/control conditions and during leaf development were investigated.

Real-Time PCR on Mature miRNAs

Total RNA was extracted from leaf tissues, pooling at least 90 plants (using the same leaf sections as those used for small RNA libraries preparation) using a 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 by 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 the 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 each correspond to the entire sequence of the miRNAs tested; all primers used are listed in Supplemental Table 11. Reaction efficiencies of RT–qPCR assays for each individual miRNA/primer were determined using a fourfold 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 (Supplemental Table 11). Relative quantification of each miRNA tested was calculated from Ct value, using the 2^(-ΔΔCt) method, directly with the BioRad CFX Manager Software.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant* Online.

FUNDING

This work was supported by grants from Scuola Superiore Sant’Anna’s Doctoral Programme in Agrobiology; from the Fondo per gli Investimenti della Ricerca di
Base-Programma Futuro in Ricerca 2010 (no. RBFR10SDOV to E.M.) of the Italian Ministero Istruzione Università e Ricerca; the Interuniversity AttractionPoles Programme (Belgian Network BARN ‘Growth and Development of Higher Plants’; IUAP VI/33), initiated by the Science Policy Office of the Belgian State, the ‘Bijzonder Onderzoeksfonds Methusalem Project’ (no. BOF08/01M00408) and the Multidisciplinary Research Partnership ‘Bioinformatics: From Nucleotides to Networks’ Project (no. 01MR0310W) of the Ghent University. No conflict of interest declared.

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