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Characterization and expression pattern analysis of microRNAs in wheat under drought stress

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Abstract

Plant microRNAs (miRNAs) play important roles in regulating plant growth, development, and responses to abiotic stresses. In this study, 38 miRNAs (TaMIRs) from wheat (*Triticum aestivum* L.), 36 from the miRBase database, and two from our previous work were characterized and subjected to an expression pattern analysis under normal conditions and a drought stress. A semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR), real-time quantitative PCR (qPCR), and small RNA blot analyses revealed that two TaMIRs (TaMIR1120 and TaMIR1123) were root-predominant and two TaMIRs (TaMIR1121 and TaMIR1134) were leaf-predominant. Seven TaMIR precursors showed altered expressions after the drought; of these, TaMIR1136 was upregulated, whereas TaMIR156, TaMIR408, TaMIR1119, TaMIR1129, TaMIR1133, and TaMIR1139 were downregulated. These seven drought-responsive TaMIRs showed dose-dependent and typical temporal expression patterns during drought induction, and they gradually returned back under the normal growth conditions. The drought-responsive and the tissue-predominant TaMIRs had varying numbers of target genes. Randomly selected target genes exhibited opposite expression patterns to their corresponding TaMIRs suggesting that they were regulated by distinct TaMIRs through a post-transcriptional cleavage. The target genes regulated by drought-responsive and tissue-predominant TaMIRs are involved in various cellular processes, such as signal transduction, transcriptional regulation, primary and secondary metabolisms, development, and defense responses. These results provide a novel insight into the miRNA-mediated responses of wheat to drought stress.

Additional key words: organ specific expression, qPCR, target genes, *Triticum aestivum*.

Introduction

MicroRNAs (miRNAs) are 19 to 24 nt non-coding RNAs that are critical to plant growth and development, as well as plant responses to diverse environmental stimuli through the post-transcriptional gene regulation pathway. A subset of biological processes is mediated in plants by miRNAs *via* a target gene mRNA cleavage or a translation-associated repression using the RNA-induced silencing complex (RISC) (Liu *et al*. 2008, Zhang *et al*. 2008, Bartel *et al*. 2010, Sunkar 2010, Ferreira *et al*. 2011, Xu *et al*. 2011b, Barrera-Figueroa *et al*. 2012).

Global water shortage caused by the increasing world population and worldwide climate alteration is becoming one of major challenges for us today (Toker *et al*. 2007). Plant tolerance to drought is a complex and quantitative trait controlled by several associated mechanisms (Fleury *et al*. 2010, Movahedi *et al*. 2012, Tian *et al*. 2012, Kang *et al*. 2013, Zhang *et al*. 2013). Recently, miRNAs involved in plant responses to drought have been identified. For instance, the conserved *Arabidopsis* miR394 and its target gene *LEAF CURLING RESPONSIVENESS* (*LCR*) are

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Abbreviations: ABA - abscisic acid; *AFB3* - auxin receptor 3 gene; *CSD* - cytosolic Cu/Zn superoxide dismutase genes; DRE - dehydration-responsive element; EST - expressed sequence tag; MS - Murashige and Skoog; PEG - polyethylene glycol; Pi - inorganic phosphate; qPCR - real-time quantitative PCR; RISC - RNA-induced silencing complex; RT-PCR - reverse transcriptase polymerase chain reaction; TaMIR - *Triticum aestivum* microRNA.
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involved in the regulation of plant response to salt and drought stresses in an abscisic acid (ABA)-dependent manner. Silencing *LCR* mRNA by miR394 is essential to maintain a certain phenotype favorable for the adaptive response to above abiotic stresses (Song *et al*. 2013). *Arabidopsis MIR168* is confirmed to control *ARGONAUTE1* (*AGO1*) homeostasis during ABA treatment and abiotic stress responses. *AGO1* transcription activity increases under ABA and drought treatments suggesting that a transcriptional elevation of *MIR168a* is required for maintaining a stable *AGO1* transcript level during stress response (Li *et al*. 2012b). miR393, a conserved miRNA identified in *Arabidopsis*, *Oryza sativa*, *Medicago truncatula*, and *Pinguicula vulgaris*, is upregulated during drought stress (Sunkar 2010). Five sugarcane miRNAs (miR164, miR394, miR397, miR399, and miR528) are differentially expressed when the plant is subjected to drought (Ferreira *et al*. 2011). Similarly, 44 of the 157 miRNAs in cowpea that belong to 89 families are drought-associated, and 30 of them are upregulated and 14 downregulated by drought (Barrera-Figueroa *et al*. 2012). Furthermore, a distinct abiotic stress-responsive module comprising miR393 and two auxin receptor gene homologs (*OsTIR1* and *OsAFB2*) has been defined in rice (Xia *et al*. 2010). These findings suggest that distinct miRNAs are critical in regulating plant responses or tolerance to drought.

Several studies focused on the characterization and analysis of miRNAs, and the prediction of miRNA target genes in wheat have been conducted (Xin *et al*. 2010, Tang *et al*. 2012, Wang *et al*. 2013). Of these, Xin *et al*. (2010) have identified a diverse set of miRNAs that are responsive to powdery mildew infection and heat stress in wheat. By sequencing small RNA libraries obtained from spike tissues of the thermosensitive male sterile (TGMS) line under cold and control conditions, Tang *et al*. (2012) revealed that 78 unique miRNAs are involved in mediating the wheat male sterility of the TGMS line under the control conditions. Although several investigations on miRNA regulated-abiotic stress responses have been reported in wheat, wheat miRNAs associated with drought stress are largely unknown. In the present study, 38 *T. aestivum* miRNAs (TaMIRs), 36 from the miRBase database, and 2 identified in our previous work were characterized and their expression patterns were analyzed under a drought stress. Our results provide insights into miRNA-mediated drought-responsive mechanisms in wheat.

Materials and methods

Plants and stress treatment: Wheat (*Triticum aestivum* L.) cv. Shixin828 seedlings were cultured hydroponically in a growth room as described by Sun *et al*. (2012). The seedlings in the third-leaf stage cultured in a Murashige and Skoog (MS) medium were transferred into the same medium supplemented with 10 % (m/v) polyethylene glycol (PEG-6000) to simulate a drought stress. After 24-h drought induction, roots and leaves of the treated and control plants were collected to determine the expression patterns of TaMIRs. To examine the expression pattern of drought-responsive TaMIRs under different drought stresses, the seedlings were transferred to MS media containing 5, 10, and 15 % PEG for 24 h. Further, the seedlings were subjected to an MS solution supplemented with 10 % PEG for 48 h and roots were collected at different time points: 0 (CK, before the treatment), 12, 24, and 48 h. Then the seedlings were transferred to MS solutions without PEG and roots were collected during recovery at time points 12, 24, and 48 h. Moreover, these roots were also used to determine the target gene expression patterns.

Characterization and expression pattern analysis of TaMIRs: A total of 38 wheat TaMIRs were subjected to a characterization and expression pattern analysis in this study. Among the TaMIRs, 36 were obtained from the miRBase database (http://microrna.sanger.ac.uk/sequences/index.shtml) and 2 (TaMIR414 and TaMIR1216) were obtained from our previous work. The names and corresponding accession numbers of the TaMIRs are shown in Table 1 Suppl.

Expression pattern analyses of the TaMIR precursors under normal growth and drought stress conditions: Total RNA was extracted from samples using a *TRIzol* reagent (*Invitrogen*, Carlsbad, USA) according to the manufacturer's instructions. The total RNA after a DNase treatment was then polyadenylated using a *poly(A) tailing kit* (*Ambion*, Bubendorf, Switzerland) according to the manufacturer's suggestions. The polyadenylated miRNAs were then reverse-transcribed into cDNAs using *Superscript II* reverse transcriptase (*Invitrogen*). The transcripts of the tested TaMIR precursors from the roots and leaves in the control group (CK) and in various drought-related samples were detected based on a semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) and real-time quantitative PCR (qPCR) using their specific primers (Table 1 Suppl.). For semiquantitative RT-PCR analysis, thermal cycling conditions were as follows: 94 °C for 5 min followed by 28 cycles of denaturation at 94 °C for 1 min, annealing at different temperatures (Table 1 Suppl.) for 30 s, an extension at 72 °C for 30 s, and a final extension of 72 °C for 10 min. For qPCR analysis, aliquots of cDNA used in the semiquantitative RT-PCR analysis were also used as templates. The thermal cycling reactions were conducted in an *ABI 7900HT* real-time PCR system (*Applied Biosystems*, Foster City, USA) supplemented with an *miScript SYBR Green* PCR kit (*Qiagen*, Duesseldorf, Germany). The expressions of TaMIR precursors were determined according to the $2^{-\Delta\Delta CT}$ method (Livak *et al.* 2001). During the semi-quantitative RT-PCR and qPCR analyses, *tubulin*, a constitutively expressed gene in wheat,

was used as internal standard to normalize the transcripts of TaMIR precursors.

Small RNA blot analysis of drought-responsive and tissue-predominant TaMIRs: A small RNA blot analysis was performed to confirm the expression patterns of the identified drought-responsive and tissue-predominant TaMIRs in the PCR analysis. Total RNA (30 μg) derived from the roots and leaves from the CK and those treated for 24 h with 10 % PEG were resolved on 15 % (m/v) polyacrylamide-urea gels and transferred onto *Genescreen Plus* membranes (*Perkin Elmer*, Boston, MA, USA). Oligonucleotides complementary to the mature TaMIRs (Table 1 Suppl.) were amplified using PCR from clone plasmids harboring the reverse primary TaMIR sequences, and were conjugated with [γ-^{32}P]ATP and T4 kinase. The membranes were incubated with a labeled probe (5.5×10^{-6} Bq of a hybridization buffer) prior to visualization using phosphorimaging according to the conventional approach. Briefly, after pre-hybridization for 1 h, the membranes were then hybridized with a solution containing the labeled probes of tested TaMIRs at 38 °C for 16 h. The membranes were washed four times [two times with 1× saline sodium citrate (SSC) and 0.1 % (m/v) sodiumdodecyl sulphate (SDS) for 20 min and two times with 0.5× SSC and 0.1 % SDS for 50 min] at 50 °C. The washed membranes were exposed to an X-ray film for 48 h and then subjected to signal detection using a phosphorimager (*GE Healthcare*, Stockholm, Sweden). U6 RNA from wheat was used as internal standard to normalize the mature TaMIR transcripts.

Identification of target genes regulated by drought-responsive and tissue-predominant TaMIRs: An online tool *psRNATarget* (*Plant microRNA Potential Target Finder*; http://plantgrn.noble.org/psRNATarget/), a bio-informatics approach for identification of putative target genes regulated by plant microRNAs, was used to identify the target genes of drought-responsive and tissue-predominant TaMIRs. The drought-responsive and root- or leaf-predominant TaMIRs served as separate queries for *BLAST* searches against the wheat-expressed sequence tag (EST) database (*TIGR Wheat Gene Index 9*). The G:U pairs, indels, and other mismatches between the sequence of each TaMIR and its putative target gene were set up to be 3, 5, 1, and 3, respectively. Functional groups of the target genes were determined based on the annotations of the wheat EST database and *BLAST* search analysis.

Expression pattern analysis of the target genes regulated by drought-responsive and tissue-predominant TaMIRs: Seven putative target genes regulated by the drought-responsive TaMIRs and four regulated by tissue-predominant TaMIRs were randomly selected to determine their expression patterns under the normal growth conditions and drought stress. The seven target genes regulated by the drought-responsive TaMIRs were *squamosa promoter-binding-like protein 16* (accession number AL810223, regulated by TaMIR156), *ORF1 protein* (accession number TC395266, regulated by TaMIR408), *DEAD/DEAH box helicase* (accession number CK214996, regulated by TaMIR1119), *major facilitator family transporter* (accession number TC441451, regulated by TaMIR1129), *calmodulin-like protein* (accession number BE516586, regulated by TaMIR1133), *endo-1,4-beta-glucanase* (accession number TC399881, regulated by TaMIR1136), and *transcription factor Myb1* (accession number TC397585, regulated by TaMIR1139). The four putative target genes regulated by tissue-predominant TaMIRs were *protein translation factor SUI1* (accession number TC405470, regulated by TaMIR1120), *catalase-1* (accession number TC372427, regulated by TaMIR1121), *WRKY DNA binding domain* (accession number TC379745, regulated by TaMIR1123), and *receptor-like protein kinase* (accession number TC402631, regulated by TaMIR1134). Expression patterns of the target genes regulated by drought-responsive TaMIRs in roots were determined at 0 h (normal condition, CK) and after 12, 24, and 48 h of the drought stress, whereas the target genes regulated by tissue-predominant TaMIRs in roots and leaves were detected in CK and after 24 h of the drought stress, based on the semiquantitative RT-PCR and qPCR analyses. Primers used to analyze the target genes are listed in Table 2 Suppl. The RT-PCR and qPCR programs, as well as the expression levels in qPCR were the same as for detecting the expression patterns of the TaMIRs.

Statistical analysis: To ensure that the results are reproducible, the RT-PCR and qPCR analyses for each sample were performed in triplicate. The mean expression of the TaMIRs and of the corresponding target genes, and corresponding standard errors were calculated and analyzed using the *Statistical Analysis System software* (*SAS Corporation,* Cory, NC, USA).

Results

Under the normal growth conditions, the 38 TaMIR precursors exhibited different expression groups in roots: high, intermediate, low, and no transcripts. The high expression group included 16 members, namely, TaMIR156, TaMIR159a, TaMIR399, TaMIR414, TaMIR444, TaMIR855, TaMIR857, TaMIR1117, TaMIR1118, TaMIR1119, TaMIR1125, TaMIR1128, TaMIR1129, TaMIR1130, TaMIR1138, and TaMIR1139. The intermediate expression group included eight members, namely, TaMIR160, TaMIR164, TaMIR167, TaMIR174, TaMIR408, TaMIR1135, TaMIR1136, and TaMIR1216. The low expression group included 10 members, namely, TaMIR159b, TaMIR841, TaMIR1120, TaMIR1123, TaMIR1126, TaMIR1127, TaMIR1131,

TaMIR1132, TaMIR1133, and TaMIR1137. The no transcripts of TaMIR1121, TaMIR1122, TaMIR1124, and TaMIR1134 were detected (Fig. 1 and Fig. 1 Suppl.). The TaMIR precursors in leaves showed comparable transcript abundance as those in roots and they were similarly classified, except for TaMIR1120 and TaMIR1123, the transcripts of which were not detected, and TaMIR1121 and TaMIR1134 which were classified into the low expression group (Fig. 1 and Fig. 1 Suppl.). These results suggest that distinct wheat TaMIRs were involved in the regulation of plant growth under the normal growth conditions. The root-predominant expression of TaMIR1120 and TaMIR1123 and the leaf-predominant expression of TaMIR1121 and TaMIR1134 were confirmed using small RNA blot analyses (Fig. 2*A,B*), which suggest that they possibly regulated the processes in a tissue-dependent manner.

The expression pattern analysis of TaMIR precursors reveals that seven TaMIRs were responsive to the drought stress. These TaMIR precursors exhibited an altered expression upon drought induction compared with those under the normal growth conditions. Among these TaMIRs, TaMIR1136 was upregulated, whereas TaMIR156, TaMIR408, TaMIR1119, TaMIR1129, TaMIR1133, and TaMIR1139 were downregulated (Fig. 1 and Fig. 1 Suppl.), which was confirmed *via* the Northern blot analysis (Fig. 2*A,B*). Therefore, these TaMIRs were transcriptionally regulated by the drought stress and were possibly involved in plant responses or tolerance to this stressor.

The TaMIR precursor transcripts in roots and leaves at 0 h (CK, before the treatment) and 24 h were detected under the different drought treatments. TaMIR1136, a drought-upregulated TaMIR, showed a gradual increase in

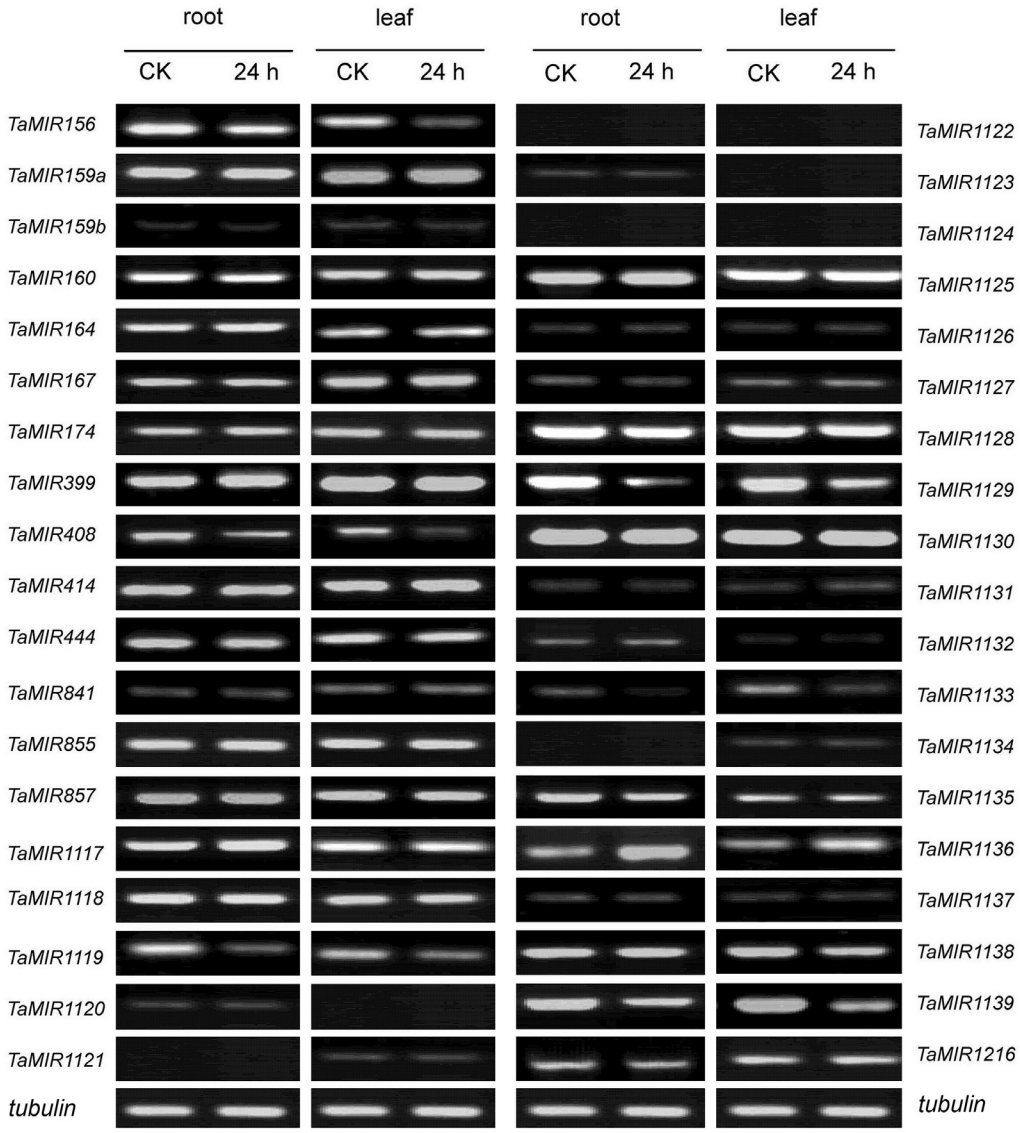

Fig. 1. Expression patterns of TaMIRs in roots and leaves detected by semiquantitative RT-PCR under control conditions (CK) and a PEG-induced drought stress (PEG).

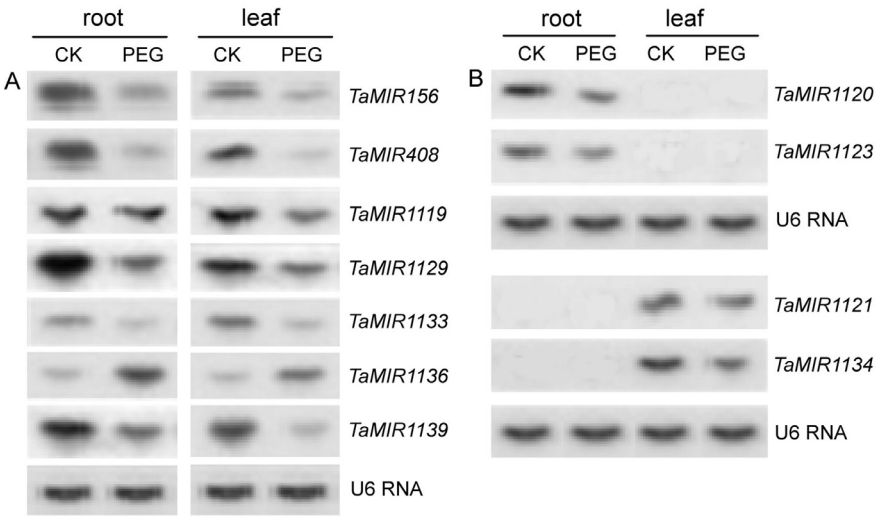

Fig. 2. The small RNA blot analysis of drought-responsive and tissue-specific TaMIRs in roots and leaves under control conditions (CK) and a drought stress (PEG). *A* - drought-responsive *TaMIRs*, *B* - tissue-specific *TaMIRs*; U6 RNA - internal standard.

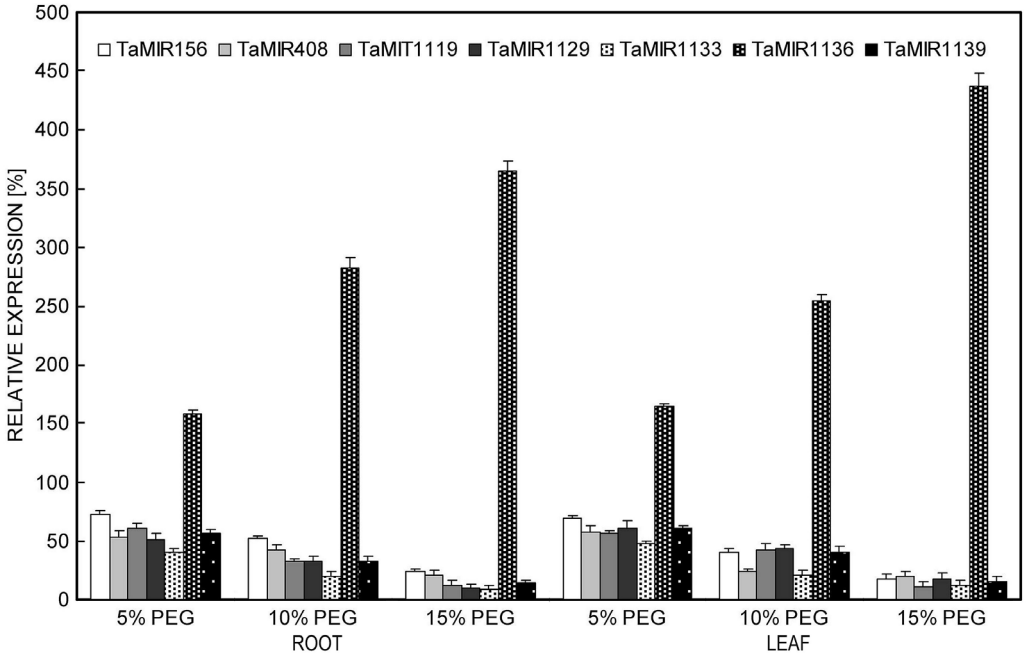

Fig. 3. Expression patterns of drought-responsive TaMIRs in roots and leaves under different drought stresses induced by PEG. The relative expression is the ratio between the expression under a drought stress and under control conditions. The *bars* represent SE of three independent assays of qPCR.

transcription in roots and leaves along with the increasing PEG concentrations. By contrast, the downregulated TaMIRs displayed a gradual decrease in transcripts in roots and leaves with the increasing PEG concentrations (Fig. 3 and Fig. 2 Suppl.). These results confirm that the transcripts of drought-responsive TaMIRs were largely regulated by the drought with a dose dependent manner.

The transcripts of drought-responsive TaMIR precursors in roots were detected at 0 h (CK, before the treatment), 12, 24, and 48 h after drought induction with 10 % PEG, as well as at 12, 24, and 48 h of recovery under the normal growth conditions. The results indicate that TaMIR1136 gradually increased the transcription with increasing exposure to drought, reaching the highest expression at 48 h. The expression of TaMIR1136 in roots decreased and during recovery reached values similar to those in CK after 48 h (Fig. 4 and Fig. 3 Suppl.). By contrast, TaMIR156, TaMIR408, TaMIR1119, TaMIR1129, TaMIR1133, and TaMIR1139 gradually decreased in roots during exposure to the drought stress, reaching their lowest expression after 48 h of the treatment. The transcription of these TaMIR precursors gradually increased when subjected to recovery and the expressions were similar to those in CK after 48 h (Fig. 4

and Fig. 3 Suppl.). These results suggest that drought-responsive TaMIRs were transcriptionally regulated by drought following a typical temporal expression pattern.

The target genes regulated by seven drought-responsive and four tissue-predominant TaMIRs were predicted: TaMIR1136 had 1 target gene, TaMIR156 had 15, TaMIR408 had 1, TaMIR1119 had 21, TaMIR1129 had 2, TaMIR1133 had 14, and TaMIR1139 had 7 target genes. Similarly, the root-predominant TaMIR1120 had 6 target genes and TaMIR1123 had 8, whereas the leaf-predominant TaMIR1121 had 5 target genes and TaMIR1134 had 14. The accession numbers of the target genes, score values between TaMIRs and their corresponding target genes, pairing position sequences in the target genes that correspond to their TaMIR, and the putative names of the target genes are shown in Table 3 Suppl. These results suggest that the drought-responsive and tissue-predominant TaMIRs performed their functions possibly through the transcriptional regulation of their distinct target genes.

One target gene of each drought-responsive or tissue-predominant TaMIR was randomly selected for a further expression analysis under the normal growth and under the drought. It was observed that the expression patterns of the target genes were opposite those of the TaMIRs. *Endo-1,4-beta-glucanase* was down-regulated during drought induction with the transcription gradually decreasing with increasing drought induction, approaching the lowest expression at 48 h. By contrast, *cytochrome P450*, *ORF1*, *DEAD/DEAH box helicase*, *major facilitator family transporter*, *calmodulin-like protein*, and *transcription factor Myb1* were upregulated during stress progression, with the transcription gradually increasing with prolonged exposure to the drought and reaching the highest expression after 48 h of the treatment (Figs. 5, 6, and Fig. 4 Suppl). Similarly, *protein translation factor SUI1* and *WRKY DNA binding domain*, root-predominant TaMIR regulating target genes, had fewer transcripts in roots and more transcripts in leaves. By contrast, *catalase-1* and *receptor-like protein kinase*, leaf-predominant TaMIR regulating target genes, had fewer transcripts in leaves and more transcripts in roots (Figs. 5, 6, and Fig. 4 Suppl.). These results suggest that the transcripts of aforementioned target genes were post-transcriptionally regulated by distinct drought-responsive or tissue-predominant TaMIRs. The drought-responsive TaMIRs and their target genes could comprise distinct miRNA/target modules to putatively regulate plant responses to drought.

The functions of drought-responsive TaMIRs are classified into the following groups: signal transduction, transcriptional regulation, RNA metabolism, primary metabolism, secondary metabolism, trafficking, development, and defense response (Table 4 Suppl.). The functions of tissue-predominant TaMIRs are classified into

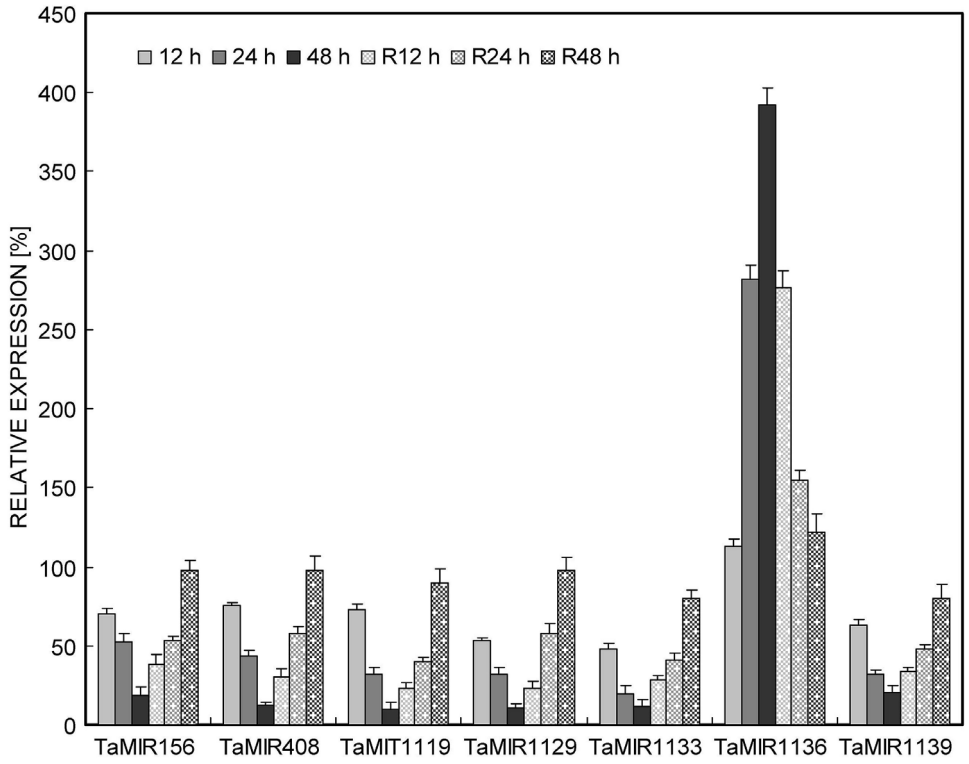

Fig. 4. Expression patterns of drought-responsive TaMIRs in roots and leaves during drought induction by PEG and subsequent recovery. The relative expressions are ratios between the expressions under the drought stress or recovery conditions and those under the normal growth conditions. The *bars* represent SE of three independent assays of qPCR. R12 h, R24 h, and R48 stand for 12 h, 24 h, and 48 h of recovery, respectively.

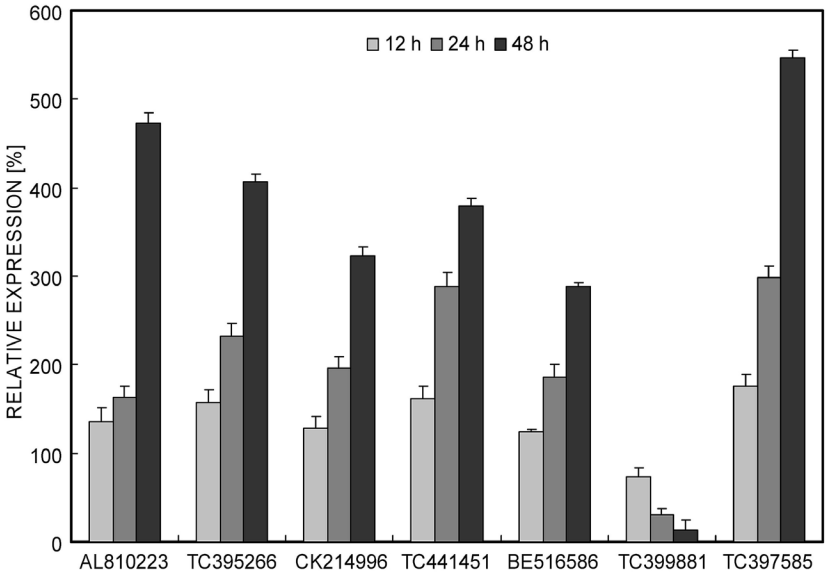

Fig. 5. Expression patterns of target genes regulated by drought-responsive TaMIRs at different times of drought induction. The *bars* represent SE of three independent assays of qPCR. AL810223 - *squamosa promoter-binding-like protein 16*, TC395266 - *ORF1 protein*, CK214996 - *DEAD/DEAH box helicase*, TC441451 - *major facilitator family transporter*, BE516586 - *calmodulin-like protein*, TC399881 - *endo-1,4-beta-glucanase*, and TC397585 - *transcription factor Myb1*.

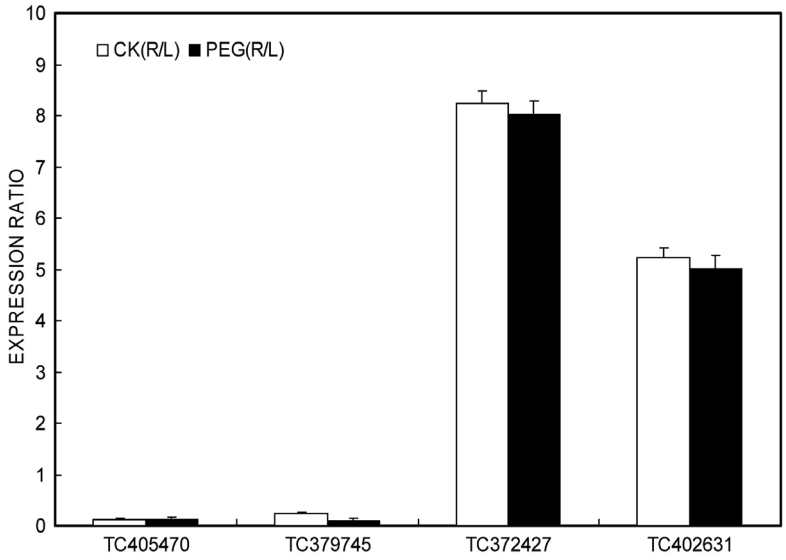

Fig. 6. Expression patterns of target genes regulated by the root- and leaf-predominant TaMIRs. The *bars* represent SE of three independent assays of qPCR. CK and PEG stand for the normal growth conditions and drought stress, respectively. R/L stands for root divided by leaf. TC405470 - *protein translation factor SUI1 homolog*, TC379745 - *WRKY DNA binding domain*, TC372427 - *catalase-1*, and TC402631 - *receptor-like protein kinase*.

the following groups: signal transduction, transcriptional regulation, post-translational modification, protein degradation, primary metabolism, secondary metabolism, development, and defense response (Table 5 Suppl.). In addition, 21 target genes regulated by drought-responsive TaMIRs have unknown functions, whereas 13 remain unannotated (Table 4 Suppl.). Similarly, 11 target genes regulated by tissue-predominant TaMIRs have unknown functions, whereas 4 remain unannotated (Table 5 Suppl).

Discussion

miRNAs have important functions in diverse biological processes (Kong *et al*. 2010, Kayihan *et al*. 2012, Zhang *et al*. 2013). A subset of miRNAs that respond to drought stress has been identified in different plant species, such as

Arabidopsis, *Medicago truncatula*, rice, sugarcane, and cowpea (Ferreira *et al*. 2011). Distinct miRNA/target modules that regulate plant responses to drought have recently been established (Xia *et al*. 2010). In *Triticum turgidum* ssp. *dicoccoides*, the ancestor of domesticated *Triticum turgidum* ssp. *durum*, thirteen miRNAs including miR1867, miR896, miR398, miR528, miR474, miR1450, miR396, miR1881, miR894, miR156, miR1432, miR166, and miR171 are differentially regulated by drought (Kantar *et al*. 2011). The miR156 members are regulated by drought in both wild and domesticated wheat suggesting that this miRNA family is conserved. However, most drought-responsive miRNA members identified in domesticated wheat are different from those identified in wild wheat indicating that these miRNA members have altered during the evolution of wheat species (Kantar *et al*. 2011). In the current study, seven miRNAs including TaMIR1136, TaMIR156, TaMIR408, TaMIR1119, TaMIR1129, TaMIR1133, and TaMIR1139 were identified to be drought-responsive. In this study, we found that the drought-responsive TaMIRs exhibited obvious dose-dependent patterns suggesting that they are associated with plant response or tolerance to drought by regulating the putative target genes *via* post-transcriptional or translation repression mechanisms.

Drought stress signaling has two established pathways: abscisic acid (ABA)-dependent and ABA-independent (Shinozaki *et al*. 1997). Most of the key genes in these drought-responsive pathways have been identified, such as dehydration-responsive element (DRE)-binding protein/ C-repeat-binding factor, ABA-binding factor, MYC, and MYB (Seo *et al*. 2009, Umezawa *et al*. 2010, Maruyama *et al*. 2012). Stress-responsive *cis*-acting regulatory elements, such as ABA-responsive element and DRE (Gómez-Porras *et al*. 2007, Maruyama *et al*. 2012), together with the motifs GCC-box *REGION1OSOSEM* (*CSGCGGCGGC*), *SBOXATRBCS* (*CCCCCTCC*), and *GAGA8HVBKN3* (GGGAGGGAG) (Zhang *et al*. 2012), have important functions in regulating gene transcription under drought stress. However, the transcriptional regulation of the drought-responsive TaMIRs and the potential roles of putative *cis*-acting regulatory elements in regulating the TaMIR transcription under drought stress are unknown. Cloning these TaMIR promoters and further identification of the *cis*-regulatory elements associated with the response to drought stress, as well as determination of the roles of these elements through a promoter-reporter system can provide further insight into the mechanisms underlying the response of TaMIRs to drought.

Several *cis*-regulatory elements function in regulating genes that are specifically expressed in roots and leaves. The *cis*-element RHE controls gene expression in roots, especially with root hair cell-predominant patterns (Won *et al*. 2009, Bruex *et al*. 2012). By contrast, some important leaf *cis*-acting regulatory elements (motifs) are only present in the promoters of genes that are preferentially expressed in leaves, including GCC core, CGACG element, SURECOREATSULTR11 (motif GAGA and CACGTG), BIHD1OS and WRKY71OS (motif ATGTCA) (Xu *et al*. 2011a), and the conserved *CATGCATGCA* sequence and *AGCTAGCTAG* element (Xu *et al*. 2011a, Zhang *et al*. 2012). In the current study, two root-predominant TaMIRs and two leaf-predominant TaMIRs were identified. Identifying their *cis*-regulatory elements and determining their function can help us understand the transcription mechanisms of these TaMIRs.

Plant miRNAs cleave the target gene transcripts in a sequence-specific manner by pairing their mature sequence with the coding regions of their target mRNAs (Millar *et al*. 2005, Ram *et al*. 2013, Vidal *et al*. 2013, Zhao *et al*. 2013). In this study, different numbers, from 1 in TaMIR1136 and TaMIR408 to 21 in TaMIR1119, of target genes were putatively regulated by tissue-predominant or drought-responsive TaMIRs. The tested target genes showed opposite expression patterns to the corresponding TaMIRs suggesting that they were regulated by TaMIRs through a post-transcriptional regulation. Our results are consistent with previous findings showing that animal miRNAs repress gene expression mainly by inhibiting translation or by promoting mRNA degradation (Dalmay 2013), and plant miRNAs negatively regulate target genes primarily by mediating RNA cleavage (Voinnet 2009, Li *et al*. 2012a). Recently, parts of target genes in plants were also observed to be regulated by miRNAs through translation repression (Yang *et al*. 2012). Although the target genes in this study indicate that they were regulated through a transcriptional cleavage, we cannot exclude that other genes were mediated by distinct TaMIRs through a translation repression mechanism. A systematic comparison analysis of the transcripts and protein expression of target genes under normal growth conditions and drought can further reveal the regulatory mechanisms of these TaMIRs.

Many biological processes in plants are accomplished through the transcriptional regulation of distinct transcription factors by their corresponding miRNAs (Seo *et al.* 2009). Recently, the target genes regulated by plant miRNAs have been observed to include diverse gene families, aside from those that encode transcription factors (Allen *et al*. 2005, Sunkar *et al*. 2006, Kong *et al*. 2010, Shuai *et al*., 2013, Vidal *et al*. 2013). These findings suggest that complicated gene networks are associated with plant growth and abiotic stress responses regulated by miRNA-mediated pathways. In this study, the target gene functions of drought-responsive and tissue-predominant TaMIRs could be categorized into several groups, such as signal transduction, transcriptional regulation, primary and secondary metabolisms, development, and defense responses. These results collectively confirm that plant responses to drought and their drought tolerance were mediated by miRNA/target modules associated with diverse gene functions.

A number of genes have been identified to be drought-responsive. In this study, although a lot of the target genes regulated by drought-responsive TaMIRs have not been annotated or functionally characterized, part of them seems to be involved in drought response given

that their putative homologs are induced by drought or are functional in plant drought tolerance. For example, the rice transcription factor squamosa promoter-binding-like 9 which interacted with miR408, a miRNA member transcriptionally regulated by cytoplasmic Ca^{2+}, may be associated with its drought response (Mutum *et al*. 2013). ZoCDPK1, a calcium-dependent protein kinase (CDPK) in ginger that harbors the conserved EF-hand loops of calmodulin-like domain, is rapidly induced by drought as well as by high salt stress and jasmonic acid treatment. Overexpression of *ZoCDPK1* confers tobacco improved drought tolerance (Vivek *et al*. 2013). QsMYB1.2, a MYB type transcription factor in cork oak, is transiently up-regulated after drought treatment (Almeida *et al*. 2013). EXTRACELLULAR PEROXIDASE 2 (CaPO2), a peroxidase isoform in *Capsicum annuum* that plays a role in local and systemic reactive oxygen species bursts and disease resistance, is also induced by drought and enhances *Arabidopsis* tolerance to drought (Choi *et al*. 2012). Thus, the target genes regulated by drought-responsive TaMIRs in this study, such as the transcription factor members of *squamosa promoter-binding-like protein 2*, *squamosa promoter-binding-like protein 3*, *squamosa promoter-binding-like protein 11*, and *squamosa promoter-binding-like protein 16* regulated by TaMIR156, *calmodulin-like protein* regulated by TaMIR1133, *transcription factor Myb1* and *chloride peroxidase* regulated by TaMIR1139, are supposed to be critical regulators in drought responses and tolerance in plants. These drought-responsive miRNAs and their regulated transcription factor as well as other putative target genes could act as putatively distinct drought-responsive modules that play critical roles in mediating plant responses and tolerance to drought stress. Further investigation of the molecular mechanisms how these modules functions in mediating plant tolerance to drought could provide an insight into miRNA-mediated drought-responsive mechanisms. Accordingly, our findings in this study are helpful for the development of novel miRNA-targeting modules associated with drought responses in wheat.

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