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Regular paper

Barley primary microRNA expression pattern is affected by soil water availability

Aleksandra Swida-Barteczka[1], Katarzyna Kruszka[1], Aleksandra Grabowska[1], Andrzej Pacak[1], Artur Jarmolowski[1], Marzena Kurowska[2], Iwona Szarejko[2] and Zofia Szweykowska-Kulinska[1]✉

[1]Department of Gene Expression, Institute of Molecular Biology and Biotechnology, Faculty of Biology, Adam Mickiewicz University in Poznan, Poznań, Poland; [2]Department of Genetics, Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland

MicroRNAs are short molecules of 21–24 nt in length. They are present in all eukaryotic organisms and regulate gene expression by guiding posttranscriptional silencing of mRNAs. In plants, they are key players in signal transduction, growth and development, and in response to abiotic and biotic stresses. Barley (*Hordeum vulgare*) is an economically important monocotyledonous crop plant. Drought is the world's main cause of loss in cereal production. We have constructed a high-throughput Real-Time RT-qPCR platform for parallel determination of 159 barley primary microRNAs' levels. The platform was tested for two drought-and-rehydration-treated barley genotypes (Rolap and Sebastian). We have determined changes in the expression of primary microRNAs responding to mild drought, severe drought, and rehydration. Based on the results obtained, we conclude that alteration in the primary microRNA expression is relative to the stress's intensity. Mild drought and rehydration mostly decrease the pri-miRNA levels in both of the tested genotypes. Severe drought mainly induces the primary microRNA expression. The main difference between the genotypes tested was a much-stronger induction of pri-miRNAs in Rolap encountering severe drought. The primary microRNAs respond dynamically to mild drought, severe drought, and rehydration treatments. We propose that some of the individual pri-miRNAs could be used as drought stress or rehydration markers. The usage of the platform in biotechnology is also postulated.

Key words: pri-miRNA, miRNA, drought, rehydration, barley genotypes

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INTRODUCTION

MicroRNAs (miRNAs) are a class of non-coding, single-stranded small RNA molecules, usually 21 nucleotides in length. They function as regulatory molecules, expressed in a particular developmental stage or in response to environmental changes. They control development, signal transduction, and protein degradation via negative regulation of gene expression. Many miRNAs are engaged in biological pathways leading to the adaptation of numerous biotic and abiotic stress factors (Khraiwesh *et al.*, 2012; Kruszka *et al.*, 2012; Barciszewska-Pacak *et al.*, 2015). These include miRNA-driven plant response to cold (Sunkar *et al.*, 2004; Zhang *et al.*, 2009), drought (Liu *et al.*, 2008; Jian *et al.*, 2010; Pieczynski *et al.*, 2013), salt excess (Zhao *et al.*, 2009), and oxidative stress induced by heavy-metal presence or nutrient limitation (Sunkar *et al.*, 2006).

Most plant miRNAs are encoded by independent transcriptional units. MiRNA genes (*MIR*) are often very long, and many of them possess introns (Szarzyńska *et al.*, 2009, Kruszka *et al.*, 2013, Kruszka *et al.*, 2014; Alaba *et al.*, 2015). The length of the *MIR* gene also varies within a single miRNA family, as was shown for ath-miRNA160a and ath-miRNA160b. ath-MIR160a is 2034 bp long and contains an intron of 1151 bp, whereas intronless ath-MIR160b is 378 bp long (Szarzyń-ska *et al.*, 2009). Transcripts of the intron-containing *MIR* genes can be constitutively or alternatively spliced (Raczynska *et al.*, 2010; Szarzynska *et al.*, 2011; Szweykowska-Kulinska *et al.*, 2013). Additional differences within miRNA precursors result from the presence of several polyadenylation sites identified either in exons or introns. Also, alternative transcription start sites are present in the *MIR* genes, which was shown in *Arabidopsis thaliana* (Szarzyńska *et al.*, 2009; Song *et al.*, 2007). Some miRNA coding sequences are polycistronic. Such units code several miRNAs and occur in *A. thaliana*, *Oryza sativa*, and *Physcomitrella patens* (Lacombe *et al.*, 2008; Talmor-Neiman *et al.*, 2006; Zhang *et al.*, 2010). While plant *MIR* genes are typically autonomous transcriptional units, there have been many examples of intragenic loci. MiRNAs can be imbedded within the introns of protein-coding genes or non-coding RNA genes (Rajagopalan *et al.*, 2006; Brown *et al.*, 2008; Szarzynska *et al.*, 2011; Kruszka *et al.*, 2013).

The *MIR* genes are transcribed by RNAPol II into primary transcript termed primary microRNA (pri-miRNA). Pri-miRNAs possess features of all RNAPol II products, like a 5' CAP and a 3' polyA tail (Lee *et al.*, 2004). They contain a sequence folding into a hairpin structure called precursor miRNA (pre-miRNA). MiRNA, together with its partner miRNA*, are imbedded in the stem of pre-miRNA. Maturation of pri-miRNA into miRNA is a multistep enzymatic process, located in the nucleus. The main protein engaged in the microprocessing is DICER LIKE 1 (DCL1). DCL1 trims pri-miRNA hairpins and dices out miRNA/miRNA* duplexes. Pri-miRNA into miRNA processing efficiency and accuracy is facilitated by DAWDLE, SERRATE (SE), and HYPONASTIC LEAVES 1 (Yu *et al.*, 2008; Yang *et al.*, 2006; Kurihara *et al.*, 2006; Szarzyńska *et al.*, 2009). The proper processing of pri-miRNAs is further stimulated by the interaction of SE with the cap-binding complex (CBC) (Laubinger *et al.*, 2008). The HUA ENHANCER 1 (HEN1) meth-

✉e-mail: zofszwey@amu.edu.pl
Abbreviations: DH, doubled haploid; RNA-seq, RNA sequencing

yltransferase 2'-O-methylates the 3' termini of the miRNA/miRNA* duplex and protects it from 3'-5' degradation or 3' uridylation (Yu *et al.*, 2005; Yang *et al.*, 2006). Next, HASTY (HST) drives the export of the miRNA/miRNA* duplex to the cytoplasm (Park *et al.*, 2005). The miRNA strand of the duplex is then loaded into the RNA-induced silencing complex (RISC), which is then guided to the target mRNA (Vaucheret *et al.*, 2004). MiRNA* is generally degraded (Eamens *et al.*, 2009).

MiRNAs regulate gene expression at the posttranscriptional level through mRNA degradation or inhibition of translation (Bartel, 2004; Carthew & Sontheimer, 2009; Lee *et al.*, 2002). The degree of complementarity between miRNA and mRNA defines the mechanism of miRNA action. mRNAs of nearly full complementarity to specific miRNAs are targeted for degradation. Such mRNAs are cleaved in the center of the miRNA/mRNA pairing region. The resulting mRNA fragments are degraded (Llave *et al.*, 2002; Rogers & Chen, 2013). Translation suppression without mRNA cleavage is observed when the miRNA/mRNA complementarity level is low. Such miRNA guides the RISC complex to target the 3' UTR of the translated mRNAs (Brodersen *et al.*, 2008).

Arising water deficit in areas designated for crop-plant cultivation is a major cause of loss in world food production (Li *et al.*, 2009). Hence, drought is one of the world's major abiotic stress factors that lead to plant growth and development inhibition. Large areas in Poland are also coping with prolonged periods of drought (Szwed *et al.*, 2010). This has escalated in the recent decades and poses a serious environmental and economic problem. Drought is defined as a disruption of intracellular balance of water caused by a predominance of transpiration over water uptake. Water depletion causes numerous alterations in plant physiology and biochemical processes. Inhibition of gas exchange and CO_2 assimilation leads to a reduction in sugar biosynthesis. Drought-induced changes in nutrient distribution cause vegetative and eventually generative growth inhibition. Studies on the influence of drought on plants are essential for recognizing the plant rescue mechanisms.

Barley is a cereal of a great economic importance (http://www.fao.org). Its estimated world production is fourth among cereals, after wheat, rice, and maize. Barley production in Poland ranks third (after wheat and rye). It is commercially used for animal feed and human consumption, and it is the primary ingredient for beer production. Since miRNAs are considered key-regulators of gene expression at the post-transcriptional and translational levels, they are good candidates for studying the mechanisms of abiotic stress responses of crop plants. However, global mature miRNA-expression analysis requires time-consuming and expensive methods.

Despite barley's great economic importance, knowledge on its miRNAs is very limited. At this time, there are only 69 barley pre-miRNAs and 71 miRNAs deposited in the miRBase (Kozomara & Griffiths-Jones, 2014). Next-generation sequencing of small RNAs provided information about barley mature miRNA sequences (Schreiber *et al.*, 2011; Lv *et al.*, 2012). Also, it has been reported that drought affects the expression profiles of many barley miRNAs, generally toward down-regulation (Hackenberg *et al.*, 2015).

Using a previously designed and constructed quantitative Real-Time RT-PCR (RT-qPCR) mirEx 2 platform for barley's 159 pri-miRNAs (Zielezinski *et al.*, 2015), we analyzed these pri-miRNAs as indicators of the barley response to drought and re-watering. In this study, two spring barley genotypes were used (Rolap and Sebastian). The plants were subjected to altered water regimes (mild and severe drought). The pri-miRNA expression variation was also assessed after soil rehydration. The obtained results were compared between the two genotypes. Our findings revealed that the pri-miRNA expression patterns are good markers of the stress level when analyzed globally. Additionally, individual pri-miRNAs are postulated to be used as drought stress or rehydration markers.

MATERIALS AND METHODS

Plant material and growth conditions. The spring barley seeds (the Rolap genotype – DH line derived from Roland and Apex cultivars) were received from the Institute of Plant Genetics of the Polish Academy of Sciences in Poznan, Poland (Devaux *et al.*, 1992). The spring barley seeds (cultivar Sebastian) were received from the Faculty of Biology and Environmental Protection of the University of Silesia in Katowice (Poland). Barley plants were grown in a walk-in growth chamber (Conviron, Winnipeg, Manitoba, Canada), in 5L pots containing field soil mixed with sand (7:2). Soil was supplemented with a multinutrient fertilizer prior to sowing. Tillering plants were nourished with straight nitrogen fertilizer. Plants were grown at 22°C day/15°C night temperatures and 16 h day/8 h night photoperiods under 800 μmol light conditions. Plants were watered to maintain 70% SWC (Soil Water Content). Plants were subjected to drought/rehydration stress when their flag leaves appeared, stage 39–41 of the Zadoks cereal development decimal code (Zadoks *et al.*, 1974). Drought stress was applied by withholding water, and the plants were collected when the SWC dropped to 30% (mild drought) and 20% (severe drought). 30% SWC was reached 24 h after withholding water, and an additional 24 h was needed to reach 20% SWC. Rehydration to 70% SWC was applied when the SWC dropped to 10%, and the plants were collected 6 h later. The shoots of four plants treated with one of the three conditions were pooled together and treated as one biological replicate. Plants were collected in three biological replicates. For each stressed sample, control plants were collected.

DNA and RNA isolation. Genomic DNA was isolated from 1g of tissue from each genotype using a DNeasy Plant Maxi Kit (Qiagen, Hilden, Germany). DNA quantity and quality was estimated with a NanoDrop ND-1000 spectrophotometer. RNA was isolated from 100mg of tissue with 38% phenol saturated with 0.1 M sodium acetate supplemented with 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate, 5% glycerol, 0.5% sodium lauroylsarcosine, and 5 mM EDTA. To remove polysaccharides, Ambion Plant RNA Isolation Aid (ThermoFisher Scientific, Waltham, MA, USA) was added during phenol extraction. RNA was precipitated with 1 volume of 0.8 M sodium citrate in a 1.2 M sodium chloride solution and 1 volume of isopropanol. RNA concentration and quality was measured with a NanoDrop ND-1000 spectrophotometer. RNA integrity was estimated on agarose gels. Contaminating DNA traces were removed with Ambion TURBO DNase (ThermoFisher Scientific, Waltham, MA, USA).

Quantitative Real-Time RT-PCR (RT-qPCR). 3 μg of DNA-depleted RNA were reverse-transcribed with Invitrogen SuperScript III Reverse Transcriptase (ThermoFisher Scientific, Waltham, MA, USA) and 0.5 μg Oligo(dT)18 Primer (ThermoFisher Scientific,

Waltham, MA, USA). cDNA templates were 4-fold diluted. PCR of the *PHT1.1* promoter fragment was performed to control the efficiency of the removal of DNA traces from the RNA samples (Kruszka *et al.*, 2013). The cDNA purity control reactions were performed with DreamTaq DNA polymerase (ThermoFisher Scientific, Waltham, MA, USA) and two specific primers (500 nM each). RT-qPCR was performed with a 7900HT Fast Real-Time PCR System (Applied Biosystems) in 384-well plates, in 10 µl of reaction volume. Power SYBR Green PCR Master MIX (Applied Biosystems, Warrington, UK), two pri-miRNA-specific primers (final concentration of 200 nM each), and 1 µl of template were used for each reaction. Each RT-qPCR reaction was performed for three biological replicates. The barley *ADP-ribosylation factor 1-like* [GenBank: AJ508228.2] transcript fragment of 61 nt was simultaneously amplified and detected as an internal reference (Rapacz *et al.*, 2012). Expression levels were calculated with the relative quantification method ($2^{-\Delta\Delta Ct}$) as a fold-change value. The R^2 values of the analyzed data (≥0.997) were calculated with LinRegPCR software (Ramakers *et al.*, 2003). The significance of the fold-change values was tested with a T-test. Primers designed and used for validation of the pri-miRNAs levels were complementary to the stem-loop structure containing the pri-miRNA fragment. The primers used were as described in Zielezinski and coworkers (2015). The pri-miRNA names were the same as in Zielezinski *et al.*, 2015 (Table S1 at www.actabp.pl). Cycling conditions for PCR and RT-qPCR are provided in Supplementary Materials S1 (at www.actabp.pl).

RESULTS AND DISCUSSION

The study presented here concerned barley pri-miRNA expression variation under water-limiting conditions and recovery. Plants were stress treated during flag-leaf development, a developmental stage important for crop yield (Fig. 1). We have constructed a platform for the simultaneous and quantitative examination of 159 pri-miRNA expression levels. This platform was based on genomic sequences of three Hordeum vulgare cultivars (Morex, Barke, and Bowman) and tested on genomic DNA of two others (Rolap and Sebastian). Hence, we consider the pri-miRNA expression-analysis platform to be universal for domesticated barley. For 21 pri-miRNAs, we were unable to detect any expression in both, the control and stressed Rolap plants. We found that these pri-miRNAs were expressed in other abiotic stresses (data not shown) and were excluded from further analysis in experiments presented in this paper. Primers detecting 6 pri-miRNAs did not match the Sebastian cultivar. Altogether, the expression data contains 138 profiles of Rolap and 132 profiles of Sebastian miRNA primary transcripts under control, drought, and rehydration conditions. RT-qPCR is a well established technology dedicated for sensitive RNA quantification of a targeted set of genes (SEQC/MAQC-III Consortium, 2014). Other method for RNA quantification is RNA-seq. The detection sensitivity and quantification accuracy of RNA-seq depends on the read depth. Pri-miRNAs expression levels are low when compared to mRNAs. Hence, pri-miRNAs quantification with the RNA-seq method would require high coverage reads. Therefore, RT-qPCR is an optimal method for pri-miRNAs' expression analysis in terms of costs, sensitivity and quantification accuracy.

Pri-miRNA levels altered by mild and severe drought

We have grouped the pri-miRNA-relative expression patterns into three categories: (i) expression upregulated; (ii) expression unchanged; and (iii) expression downregulated. From the drought-treatment results, we concluded that pri-miRNA expression reflects the stress level applied to plants. Mild drought mostly downregulated pri-miRNAs (35 and 27 downregulated, 21 and 7 upregulated in the Rolap and Sebastian genotypes, respectively). The number of mild drought-reduced pri-miRNA expression was generally followed with severe drought in both genotypes (36 and 23 in the Rolap and Sebastian genotypes, respectively). Severe drought induced expression in 48 and 21 of pri-miRNAs in the Rolap and Sebastian genotypes, respectively. Thus,

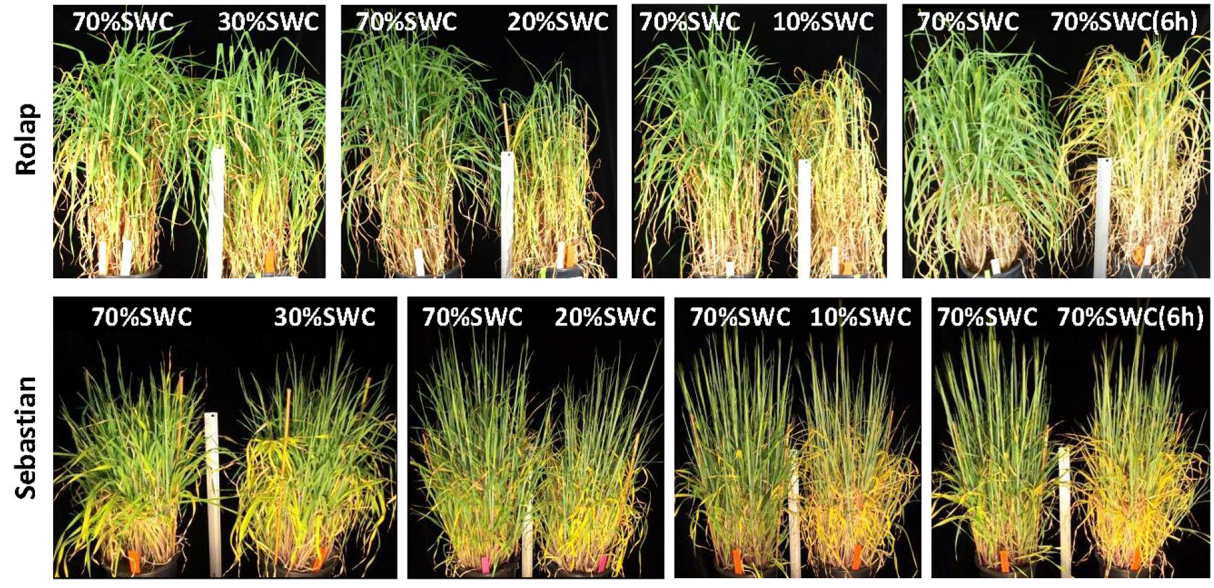

Figure 1. Control, mild drought, severe drought, and rehydration treated plants of two barley genotypes (Rolap and Sebastian).
70% SWC, control; 30% SWC, mild drought; 20% SWC, severe drought; 10%, severe drought (not tested in the study); 70% SWC (6 h), rehydration.

Table 1. General tendencies in pri-miRNA expression in two *Hordeum vulgare* genotypes (Rolap and Sebastian) altered by mild drought, severe drought, and rehydration.
↑ – upregulated, ↓ – downregulated, = – unchanged.

	mild drought			severe drought			rehydration		
	↑	=	↓	↑	=	↓	↑	=	↓
Rolap	20	25	24	38	18	21	38	25	19
Sebastian	6	40	16	11	53	8	6	51	18
both	1	57	11	10	35	15	10	35	11

while mild drought's dominant feature was a decrease in the pri-miRNA expression, severe drought stands out by the induction of pri-miRNAs. From this data, we cannot conclude whether this was due to the strength of stress or duration of the drought treatment (24 h for 30% SWC and 48h for 20% SWC). However, our studies of the mature miRNAs showed that even prolonged mild drought did not significantly influence their levels (data not shown). Under rehydration conditions, the level of 78 Rolap pri-miRNAs was affected (48 were upregulated, and the expression of 30 pri-miRNAs was decreased). The water re-supply in the Sebastian genotype upregulated 16 pri-miRNAs, downregulated 29 of them (45 pri-miRNAs altered), and did not influence the level of 86 pri-miRNAs. The re-watering study suggests that six hours of optimal water supply was not sufficient to reverse the severe-drought-induced changes in the pri-miRNA expression. Previously, comprehensive studies on pri-miRNA expression under drought treatment were conducted only in Arabidopsis (Barciszewska-Pacak *et al.*, 2015). Arabidopsis and barley pri-miRNA response to drought was similar. Mild drought mostly downregulated the ath-pri-miRNAs, while the main feature of severe drought was ath-pri-miRNAs upregulation. Summary of these analyses is presented in Table 1.

Pri-miRNA level dynamics during mild to severe drought transition

As mentioned earlier, pri miRNA expression was mainly decreased under mild drought conditions. In the Rolap genotype , we observed that 18 of the downregulated pri-miRNAs in the mild drought treated plants were also downregulated in case of severe drought (Fig. 2, Table S2 at www.actabp.pl). Surprisingly, there was a set of 17 mild drought downregulated pri-miRNAs that responded to severe drought by induction of their expression. The expression of nine of them returned to the control level under severe drought, and the remaining eight were even upregulated. Similarly, in Sebastian we identified 12 mild and severe drought downregulated pri-miRNAs and 15 in which expression was decreased under mild drought and induced during the severe drought stress.

A group of 21 pri-miRNAs was induced by mild drought in the Rolap genotype. Their expression mostly stayed upregulated under severe drought, except for one that was strongly decreased under severe drought and further under rehydration. The Sebastian cultivar showed mild drought-induced levels for only seven pri-miRNAs. Three of them were constantly induced under severe drought.

Mature miRNA expression dynamics is not always reflected by their cognate pri-miRNA expression lev-

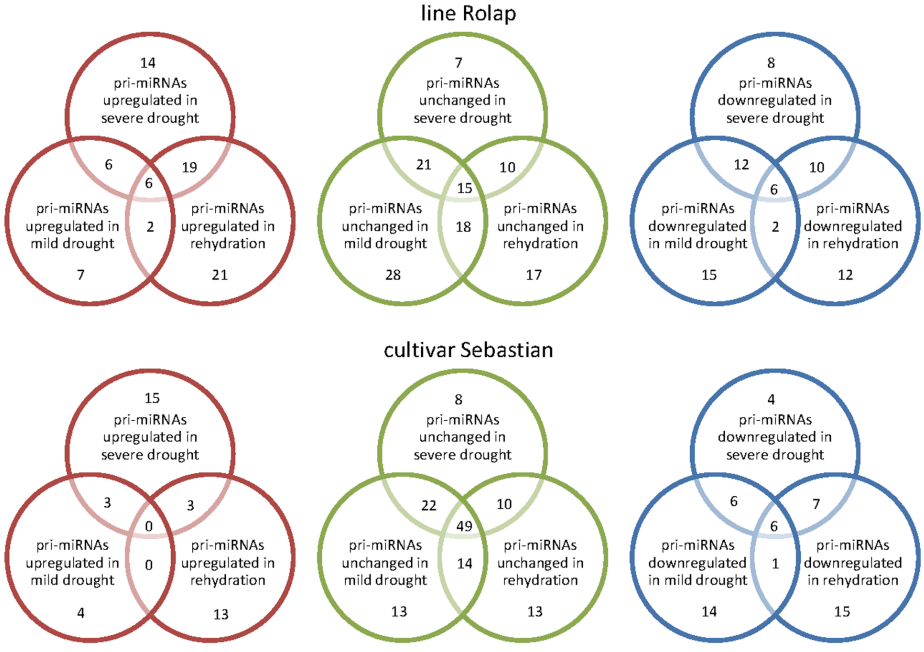

Figure 2. Diagrams showing relations between the pri-miRNA category of different treatments.
Each diagram shows one category of pri-miRNAs: upregulated, unchanged, and downregulated. The barley Rolap, and Sebastian genotypes pri-miRNA expression data are shown on separate diagram sets.

Table 2. Pri-miRNA169 expression under mild drought, severe drought, and rehydration in two *Hordeum vulgare* genotypes (Rolap and Sebastian).
RTqPCR results are shown as fold-change values. The levels of pri-miRNAs under the control conditions were assumed to be '1', and the levels of pri-miRNAs under stress conditions were quantified in relation to this standard. The up- and down-regulated pri-miRNAs were suggested by a two-tailed Student's *t*-test (***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$). Up, down-regulated pri-miRNAs are marked as red and blue, respectively (nd, not detected).

	Rolap			Sebastian		
pri-miRNA	mild drought	severe drought	rehydration	mild drought	severe drought	rehydration
169f	2.17**	5.88*	1.29	1.59	3.38*	9.41
169h	1.44	2.53	2.05*	1.21	1.80	2.68***
169d	1.13	0.84	2.56*	0.63	0.84	1.44
169a	1.18	1.30	1.31*	3.62	6.54	0.03***
169m	1.32*	0.35**	0.10***	0.89	1.20	0.36**
169e	2.43	0.12***	0.14***	0.71	0.38*	0.35**
169c	0.75**	0.75**	3.65	nd	nd	nd

els. This was thoroughly studied in Arabidopsis under many abiotic stresses (Barciszewska-Pacak *et al.*, 2015). The data presented by Barciszewska-Pacak and coworkers (2015) showed that the level of particular miRNA could not be predicted based on the level of its particular pri-miRNA. Hence, the most probable cause of miRNA expression fluctuations are changes in the pri-miRNA processing. Pri-miRNAs undergo posttranscriptional processes like splicing, alternative splicing and alternative polyadenylation site selection. Posttranscriptional pri-miRNAs' processing is correlated with mature miRNAs' expression levels (Bielewicz *et al.*, 2013; Jia & Rock, 2013). Yan and coworkers (2012) showed that heat stress induced alternative splicing event led to *Arabidopsis* pri-miRNA400 accumulation which resulted in the mature miRNA400 decrease. It was shown that during salt stress in *Arabidopsis*, in some cases, the decreased level of pri-miRNAs correlated with increased accumulation of mature miRNAs and not their complementary miRNAs* (Dolata *et al.*, 2016). This was due to AGO1 binding and stabilization of these miRNAs. Changes in pri-miRNA levels could be the result of the *MIR* expression regulation at the level of transcription. Abiotic stress response elements were computationally predicted in promoter regions of many *Arabidopsis* miRNA genes (Megraw *et al.*, 2006; Zhao *et al.*, 2013). Transcriptional regulation of *MIR* can explain the observed expression fluctuations in case of some Arabidopsis or barley pri-miRNAs. Nevertheless, drought responsive elements in the barley *MIR* are not known. An interesting observation was made in *A. thaliana* and *Solanum tuberosum*, where a consequence *of CBP80* expression-silencing was a decrease in mature miRNA159 (Pieczynski *et al.*, 2013). CBP80 and CBP20 are known to be involved in pri-miRNA maturation and affect the level of several mature miRNAs (Szarzynska *et al.*, 2009; Laubinger *et al.*, 2008; Raczynska *et al.*, 2010; Raczynska *et al.*, 2014). Downregulation of the miR159 level caused *MYB33* target gene expression upregulation and induced Arabidopsis and potato drought tolerance and hypersensitivity to ABA. As pri-miRNA biogenesis is a multistep process, there are many ways to posttranscriptionally regulate *MIR* transcripts. We concluded that drought stress-induced changes in the pri-miRNA expression pattern may also affect the level of mature miRNAs.

Severe drought-induced changes in pri-miRNA expression can be reversed during rehydration

As stated earlier, severe drought mainly induced pri-miRNA expression in the Rolap variety (Table 1). In contrast, rehydration allowed the reversion of expression of 23 severe drought-induced pri-miRNAs (out of 48) to control or even lower levels, already within six hours of rehydration (Table S2 at www.actabp.pl). The remaining 25 drought-induced pri-miRNAs remained upregulated after 6h of water re-supply (Fig. 2). Similarly, drought downregulated expression returned to control or increased levels for 20 out of the 36 pri-miRNAs.

In barley plants of the Rolap genotype, rehydration surprisingly induced the expression of 19 pri-miRNAs. Their expression was not affected by severe drought (Table S2 at www.actabp.pl). In addition, expression of 10 pri-miRNAs not affected by severe drought was reduced after water re-supply.

Interestingly, the pri-miRNA expression study for the Sebastian cultivar gave slightly different results. Of the 21 pri-miRNAs upregulated under severe drought, 17 pri-miRNA expression levels returned to their initial level of expression with rehydration. Thus, severe drought-induced pri-miRNA levels were mainly reversed by re-watering in the Sebastian cultivar. In the Rolap line, this was observed only for 23 out of 48 pri-miRNAs. The Rolap and Sebastian genotypes were never compared in terms of their water-depletion stress resistance. Moreover, neither of these genotypes were studied toward drought resistance. The differences in pri-miRNA expression may suggest drought-resistance dissimilarity between these two genotypes. Our results indicated that the Rolap line susceptibility to water supply limitations is higher than Sebastian's. Our observation was strengthened by the fact that one of the Rolap parents is the highly drought intolerant Roland cultivar (http://agris.fao.org/agris-search/search.do?recordID=PL9201081).

Of the 16 pri-miRNAs upregulated upon rehydration in Sebastian, 13 pri-miRNAs were recognized as specifically rehydration induced. Thus, rehydration additionally stimulated new changes in the pri-miRNA expression. Severe drought decreased the expression of 23 pri-miRNAs in Sebastian. The level of these pri-miRNAs returned to the initial level after re-watering in case of 10 pri-miRNAs.

Table 3. A list of pri-miRNAs identified as markers of drought-, mild drought-, or rehydration-treated barley plants.

pri-miRNA as universal drought/rehydration markers	drought		mild drought	rehydration	
	upregulated	downregulated	downregulated	upregulated	downregulated
	160f	160a	1130b	159a	164a
	167b	169e	399c	159c	2118c
	169f	166j	5049h	169h	5049b
	171b	171c		1120	6183
	2275a	172c		6212	
	399c	319b		9674b	
	399e	396c			
	5048a	398a			
	5049e	399a			
	6199	5052			
		5168			
		6176			
		6207			
		6208			
		6211			

Pri-miRNA169 expression under drought and rehydration

The MiRNA169 family was shown to be involved in drought resistance in Arabidopsis and *Solanum lycopersicum*. Water deficit or abscisic acid (ABA) treatment decreased the ath-miRNAs169a/c (Li *et al.*, 2008). These miRNAs target the mRNA of *NFYA5*, a subunit of transcription factor NF-Y. NFYA5 acts in stomata cells, decreasing transpiration in an ABA-dependent way. The overexpression of *MIR169a* had caused a higher drought intolerance in Arabidopsis. Moreover, the deletion of *MIR169a* generated a drought-resistance phenotype (Zhao *et al.*, 2016). In contrast, in *S. lycopersicum*, the drought-induced accumulation of miRNA169 and, consequently, the overexpression of miRNA169c, triggered a drought-tolerant phenotype (Zhang *et al.*, 2011). Our study showed that members of the pri-miRNA169 family were differentially regulated under drought/rehydration stress (Table 2). The MiRNA169 family members are good candidates for inducing drought tolerance in barley.

Pri-miRNAs as water limitation/re-supply markers

As drought can be correlated with several pri-miRNA expression profiles, we have proposed a set of mild drought, drought, and rehydration markers. The selected pri-miRNAs responded similarly in both of the tested genotypes (Table 3).

Severe drought upregulated the expression of 10 orthologous pri-miRNAs in Rolap and Sebastian, while 15 orthologous pri-miRNAs were downregulated in both genotypes (Table 3). These 25 pri-miRNAs are proposed to be the drought-stress markers. Mild drought could be identified by the fact that pri-miRNA1130b, pri-miRNA399c, and pri-miRNA5049h levels decreased. These precursors were only downregulated in the Rolap and Sebastian under mild drought. We propose to use 10 pri-miRNAs as markers of plant recovery from drought. Rehydration in barley induced the expression of six orthologous pri-miRNAs in Rolap and Sebastian. Profiles of these pri-miRNAs were downregulated or not affected by mild and severe drought. Other rehydration markers could be four pri-miRNAs (pri-miRNA164a, pri-miRNA2118c, pri-miRNA5049b, and pri-miRNA6183), whose levels were reduced regardless of the variety tested. This is the first study where pri-miRNAs were analyzed as stress markers.

Drought induces complex changes in plants, which leads to growth and development inhibition. Our study showed that pri-miRNAs respond strongly to drought. The usage of pri-miRNAs' RT-qPCR platform under three different water limitation/rehydration regimes revealed differential *MIR* gene reprograming among the three treatments. We recommend the primary microRNA RT-qPCR-based platform to be a universal tool for testing the strength of drought response in barley. Hence, the platform can be used to determine the drought stress levels applied to barley plants. Also, it would be beneficial to use the pri-miRNAs RT-qPCR platform in studies on barley transition between drought response and drought acclimation. MicroRNAs in barley's homeostasis under prolonged drought were not yet studied. Pri-miRNAs expression studies can be used to reveal candidates for future genetic analysis when looking for drought tolerance/resistance markers. As miRNAs mostly regulate the transcription-factor expression, they are excellent candidates for plant biotechnological studies. *MIR's* inactivation became possible with CRISPR/Cas9 technology (Zhao *et al.*, 2016). It would be interesting to recognize which miRNAs activate plant drought-rescue pathways. Genes regulated by miRNAs are subsequent objectives for such studies. The pri-miRNAs RT-qPCR platform can be also used to test barley response under other abiotic and biotic stresses.

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