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Developmental changes in barley microRNA expression profiles coupled with miRNA target analysis

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MicroRNAs are 19- to 24-nt-long single-stranded RNAs that are crucial regulators of gene expression which control plant development and response to environmental cues. We have analyzed microtranscriptomes of five barley developmental stages. Generally, during the barley development, miR168-3p and miR1432-5p levels increase while the 5'U-miR156-5p level decreases (with exception for the 2-week-old barley). We have identified two miR156-5p izomiRs (called 5'U-miR156-5p [20 nt] and 5'UU-miR156-5p [21 nt]), which were expressed differently during barley development. The 5' U-miR156-5p level decreased in 3-week-, 6-week-, and 68-day-old barley, when compared to the 1-week-old plants. Meanwhile, the 5' UU-miR156-5p level increased significantly in the 68-day-old barley plants. Moreover, only the 5' U-miR156 isomiR recognizes and guides unique transcription factor mRNAs from the Squamosa Promoter Binding Protein-Like (SPL) family. We identified many non-canonical microRNAs with changed expression levels during the barley development. Here, we present the profiles of microRNA expression characteristics for particular barley developmental stages. These analyses are accompanied by the experimental degradome analysis of miRNA targets.

Key words: barley, microRNA, deep sequencing, development

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INTRODUCTION

MicroRNAs are small molecules that act on complementary target mRNAs, leading to their degradation via the endonucleolytic activity of Argonaute (AGO). In Arabidopsis, there are ten AGO proteins (Garcia-Ruiz *et al.*, 2015). AGO proteins that contain the DDH motif can cleave target mRNAs (Baumberger & Baulcombe, 2005; Carbonell *et al.*, 2012). A microRNA bound with AGO creates RISC (RNA-Induced Silencing Complex). Hsp90 facilitates RISC assembly (Iki *et al.*, 2010). The role of microRNA is predominantly related to guiding the AGO1 protein to the proper mRNA target. Canonical cleavage of target mRNA occurs at the position located opposite the 10–11th nucleotide (counting from the guiding miRNA 5' end). In Arabidopsis, more than half of the target mRNAs are transcription factors (TFs) (Fahlgren *et al.*, 2007). The primary transcript of a microRNA is produced by RNA Pol II followed by the excision of the pre-miR stem-loop precursor by the DCL1 in the nucleus. Then, double-stranded microRNA/microRNA* duplex is excised from the pre-miR precursor, exported to the cytoplasm, and bound by the AGO protein (Mallory & Vaucheret, 2010; Schirle & MacRae, 2012). There are 325 precursors and 427 mature Arabidopsis microRNAs, and only 69 and 71 barley microRNA precursors and mature microRNAs (respectively) are deposited in the miRBase (MiRBase, release 21: June 2014; (Kozomara & Griffiths-Jones, 2011)). The nucleotide sequence, miRNA length, 5' end terminal nucleotide of microRNAs, and miR/miR* duplex structure dictate the sorting and future action of a particular microRNA (Zhang *et al.*, 2014). In Arabidopsis, a predominant fraction of microRNAs (76%) contains molecules that are 21 nt long, while the remnant microRNAs represent smaller populations: 11% – 22 nt long; 9% – 20 nt long; 3% – 24 nt long; 1% – 23 nt long; and only 0.23% – 19 nt long. Additionally, in the case of 56% of 21 nt Arabidopsis microRNAs, the first nucleotide from the 5' end is occupied by uridine, which suggests miRNA association with AGO1 and its involvement in mRNA cleavage (unpublished data, Zhang *et al.*, 2014).

Barley development is mostly described by using phenotypical features like leaf number, tiller numbers, and kernel stage (Zadoks *et al.*, 1974). In our previous work, we described pri-microRNA expression profiles in five barley developmental stages (Kruszka *et al.*, 2013). Moreover, in the mirEX 2.0 database (an integrated environment for the expression profiling of plant microRNAs), we included 140 barley pri-microRNAs and also mature microRNA expression profiles for these 5 barley developmental stages (Zielezinski *et al.*, 2015). Although the pri-miRNA and miRNA expression profiles are deposited in the mirEX 2.0 database, they were never analyzed and compared in detail in the different barley developmental stages. Here, we present a global analysis of the barley microtranscriptome profiles identifying the microRNAs specific for the various barley developmental stages. Moreover, we also identified miRNA-targeted mRNAs. In addition, we present a deeper microRNA isomiR analysis in the case of miR156-5p.

MATERIALS AND METHODS

Plant material. *Hordeum vulgare* cv. Rolap seeds were received from the Institute of Plant Genetics of the

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Abbreviations: NGS, Next Generation Sequencing

Polish Academy of Sciences (Poznań, Poland) (Devaux *et al.*, 1992). Plants were sampled in five developmental stages identified in the Zadoks scale (Zadoks *et al.*, 1974): 1-week-old plants (code 11, one leaf developed), 2-week-old plants (code 13, third leaf developed), 3-week-old plants (code 20-21, beginning of tillering), 6-week-old plants (code 32-36, stem elongation), and 68-day-old plants (code 75-77, kernels in milk ripeness). Ten whole plants for each particular developmental stage were treated as one sample.

RNA isolation. A total RNA enriched in small RNAs was isolated using the previously described approach (Kruszka *et al.*, 2013). The quality of the isolated RNA was verified using an Agilent 2100 Bioanalyzer and Nano Plant RNA assay (Agilent).

Small RNA library preparation. Four independent small RNA libraries were designed for each barley developmental stage. Three replicas were performed using an Illumina TruSeq Small RNA Library Preparation Kit (Illumina). The quantification of libraries was carried out using a Quant-iT PicoGreen dsDNA Assay kit (Molecular Probes) reagent and Tecan Infinity 200 Pro Spectrometer (Tecan). NGS was performed using a HiScan-SQ machine (Illumina). The fourth replicated library for each developmental stage was performed using a different protocol. In brief, 1 μg of total RNA was ligated to a 3' adenylated adapter using a truncated T4 RNA ligase2 (New England Biolabs). After 1 h of incubation at 22°C, an RNA 5' adapter was added with T4 RNA ligase (Ambion), ATP and incubated at 37°C for 1 hour. cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen). PCR was performed using a Phusion Hot Start High Fidelity DNA polymerase (New England Biolabs). PCR was run at 98°C for 30 sec, followed by 15 cycles at 98°C for 15 sec, 62°C for 30 sec, and 72°C for 15 sec, followed by a 5 min incubation. Products were separated electrophoretically, and the desired miRNA fraction in size was excised and eluted. The quality was tested using an Agilent Bioanalyzer DNA1000 chip and quantified using an Qubit fluorometer (Invitrogen). Each library was diluted to a target concentration for cluster generation and then loaded onto a lane of an Illumina GAiiX flow cell. The fourth library and its sequencing was performed at the BC Cancer Agency, Vancouver, BC, Canada.

Degradome. Degradome analysis (Parallel Analysis of RNA ends [PARE]) was performed using the approach used in previously published papers (German *et al.*, 2009; Alaba *et al.*, 2015).

Dataset analysis. Four replications were used for each particular barley developmental stage. Deep-sequencing data analysis was carried out using the approach described previously (Alaba *et al.*, 2015). Then the normalized counts for each sRNA were averaged from replicates and the fold change and p-value were calculated between conditions using the Student's t-test. In the case of replications where no reads were detected (0 reads), value of 1 was added to the average in order to enable fold change calculation. For further analysis of the expression pattern, we considered microRNAs that have abundance described by either $\log_2$ (Fold Change) >1 (increase higher than 2 fold) or $\log_2$ (FC) <–1 (decrease higher than 2 fold) with the p value <0.05 (Student's t-test). Barley pri-microRNA and microRNA expression profiles deposited in the mirEX 2.0 database are available at the following web page: http://www.combio.pl/mirex (Zielezinski *et al.*, 2015).

Target prediction and verification based on the degradome data. Possible target identification was performed using the psRNATarget online tool, (http://plantgrn.noble.org/psRNATarget) with default settings (Dai & Zhao, 2011). When possible, the predicted targets were verified with our data obtained from the degradome sequencing.

Barley stem-loop pre-miR structure identification. For construction of the stem-loop pre-microRNA precursors, the following sequences were used:

- pre-miR827 – morex_contig_142720 CAJW010142720 carma=2HL
- pre-miR319-5p – morex_contig_46695 CAJW0100-46695 carma=3HS
- pre-miR171-5p – morex_contig_54459 CAJW01005-4459 carma=2HL
- pre-miR1432-5p – morex_contig_137798 CAJW010-137798 carma=2HS
- pre-miR171-3p – morex_contig_2550634 CAJW0125-50634 carma=2HL
- pre-miR319-3p – morex_contig_134927 CAJW010-134927 carma=4HS
- pre-miR156-5p – morex_contig_135129 CAJW010-135129 carma=6HS

Sequences presented above were found by using the IPK BLAST Server (IPK Gatersleben – http://webblast.ipk-gatersleben.de/barley/) and the ViroBLAST online tool (Deng *et al.*, 2007). The obtained sequences were folded using Folder version 1.11 (RNAfold algorithm) software available at http://www.ncrnalab.dk/#rnafolder/rnafolder.php. The longest sequences which could form the stem-loop structures were used for pre-microRNA construction. The pre-microRNA structures with the lowest ΔG energy value were chosen.

RESULTS

Using the Illumina deep-sequencing approach, we analyzed the microtranscriptomes of five barley developmental stages with regard to phenotypic traits. For each stage, four small independent RNA libraries were constructed and sequenced. Normalized reads from a particular developmental stage were compared, and fold changes were calculated. Analyses were performed in three ways:

(i) we identified the differences in microRNA expression profiles in 2-week-, 3-week-, 6–week-, and 68-day-old barley plants, when compared to the 1-week-old barley plants (Suppl. Table 1 at www.actabp.pl);

(ii) we compared microRNA expression profiles between particular barley stages to find unique expression profiles for the selected developmental stage (Suppl. Table 2 at www.actabp.pl);

Based on the statistically significant differentially expressed microRNAs presented in Suppl. Table 1, we analyzed 14 up-regulated and 11 down-regulated microRNAs in further steps. The same sets of microRNAs were analyzed for expression profiles in the barley developmental stages compared to 1-week old barley plants (Fig. 1) and other barley developmental stages compared between each other (Fig. 2).

(iii) we analyzed potential target mRNAs for the selected microRNAs based on the performed degradome analysis. Degradome data were obtained for 68-day-old barley plants (Table 1). Characterization of the identified target mRNAs was carried out by comparisons to known nucleotide/protein sequences (Table 2).

Barley microRNAs showing different expression profiles during development

MiR168-3p and miR1432-5p are more frequent, and 5'U-miR156-5p (20 nt) is less frequent in all of the older

Table 1. Barley microRNA targets. The targets were identified based on the degradome data obtained from PARE–Seq of 68–day–old barley.
Accession no, Target id – based on Ensembl Plants accession number; length, length of the analyzed cDNA; cleavage, cleavage_site (deg_score|deg_rank) – cleavage position, degradome score, degradome ranking; * cleavage position between nucleotides with match to 10th and 11th nucleotide of microRNA counting from the 5′ end, + non canonical cleavage position; score – aln_score – alignment score between microRNA and target sequence (the lower the value, the better the score); energy, hybridization energy (kcal/mol). Only targets with the best score numbers were included in the table.

microRNA	5′ sequence 3′	nt	Accession no	length (bp)	cleavage	non canonical	score	energy
miR156–5p	ttgacagaagagagtgagcac	21	MLOC_52321.1	1607	c:871(1.54|9)*	–	2	–34.4
miR156–5p	ttgacagaagagagtgagcac	21	MLOC_62426.1	1539	c:933(0.78|11)*	c:932(2.35|5)+	2	–34.4
miR156–5p	ttgacagaagagagtgagcac	21	MLOC_11199.7	3162	c:2792(6.54|2)*	–	3	–32.6
miR156–5p	ttgacagaagagagtgagcac	21	MLOC_13032.1	1112	c:751(2.94|4)*	–	4.5	–31
miR156–5p	tgacagaagagagtgagcac	20	MLOC_37841.5	2319	c:1527(3.85|1)*	–	1.5	–33.8
miR156–5p	tgacagaagagagtgagcac	20	MLOC_52321.1	1607	c:870(2.56|7)*	c:871(1.54|9)+	1.5	–33.8
miR156–5p	tgacagaagagagtgagcac	20	MLOC_62426.1	1539	c:932(2.35|5)*	–	1.5	–34.1
miR156–5p	tgacagaagagagtgagcac	20	MLOC_61297.1	3239	c:787(1.48|5)*	–	1.5	–33.8
miR156–5p	tgacagaagagagtgagcac	20	MLOC_11199.7	3162	c:2791(15.90|1)*	c:2792(6.54|2)+	3	–31.7
miR156–5p	tgacagaagagagtgagcac	20	MLOC_13032.1	1112	c:750(20.93|1)*	c:751(2.94|4)+	3	–30.8
miR159–5p	gagctcctatcattccaatga	21	MLOC_38762.5	1004	c:607(1.69|6)*	c:608(1.69|6)+	9	–26.3
miR159–3p	tttggatgaagggagctctg	20	MLOC_75369.1	2856	c:1435(1.51|1)*	–	9.5	–28.1
miR159–3p	tttggattgaagggagctctg	21	MLOC_55324.1	1336	c:117(2.75|1)*	–	5.5	–28.6
miR159–3p	tttggattgaagggagctctg	21	MLOC_71332.2	1877	c:863(3.67|2)*	–	6	–32.5
miR159–3p	tttggattgaagggagctctg	21	MLOC_72581.1	1884	c:1417(2.28|3)*	–	10	–23.3
miR167–5p	tgaagctgccagcatgatctga	22	MLOC_58330.5	2656	c:2540(11.72|1)*	–	9.5	–31.1
miR168–5p	tcgcttggtgcagatcgggac	21	MLOC_12447.2	3053	c:249(2.52|3)*	–	8	–33.9
miR168–3p	cccgccttgcaccaagtgaat	21	MLOC_63404.6	2457	c:2226(2.60|8)*	c:2227(2.09|9)+	15	–20.2
miR169–3p	ggcggtcaccttggctagc	19	MLOC_63790.1	2838	c:2474(1.57|5)*	–	7	–25.7
miR171–5p	cggtattggtgcggttcaatc	21	MLOC_11773.1	5489	c:5188(1.68|7)*	–	14.5	–22.5
miR319–5p	agagcgtccttcagtccactc	21	MLOC_61362.3	1898	c:1842(4.75|4)*	c:1843(4.31|5)+	13.5	–24.2
miR319–3p	cttggactgaagggagctcc	20	MLOC_71332.2	1877	c:863(3.67|2)*	–	3.5	–35.7
miR390–5p	aagctcaggagggatagcgcc	21	MLOC_37712.1	2369	c:1889(2.14|7)*	–	14.5	–25.7
miR390–5p	aagctcaggagggatagcgcc	21	MLOC_33978.3	1949	c:1652(10.17|2)*	–	17.5	–25.4
miR393–5p	ttccaaagggatcgcattgat	21	MLOC_56088.1	3166	c:2471(15.19|4)*	–	4.5	–33.5
miR393–5p	ttccaaagggatcgcattgat	21	MLOC_9864.2	1973	c:1429(7.27|6)*	–	4.5	–33.5
miR396–5p	tccacaggctttcttgaactg	21	MLOC_80060.1	1053	c:753(9.59|1)*	–	3	–35.4
miR396–5p	tccacaggctttcttgaactg	21	MLOC_67201.2	2342	c:913(13.91|1)*	–	6.5	–28.8
miR408–3p	tgcactgcctcttccctggc	20	MLOC_69288.1	1039	c:861(8.16|1)*	–	1.5	–39.8
miR827–5p	ttttgttggttgtcatctaacc	22	MLOC_52144.1	2112	c:1980(3.42|9)*	–	12	–16.4
miR827–5p	ttttgttggttgtcatctaacc	22	MLOC_70149.1	2853	c:1704(1.82|1)*	–	14	–17.9
miR827–5p	ttttgttggttgtcatctaacc	22	MLOC_59423.5	3157	c:1863(16.80|1)*	–	14.5	–20.4
miR1432–5p	ttcaggagagatgacaccgac	21	MLOC_70272.1	1299	c:295(7.20|1)*	–	3.5	–39.8
miR1432–5p	ttcaggagagatgacaccgac	21	MLOC_70788.1	800	c:159(2.18|1)*	–	3.5	–35.7
miR1432–5p	ttcaggagagatgacaccgac	21	MLOC_61497.1	1339	c:1006(54.61|1)*	–	8.5	–30.2
miR1432–5p	tcaggagagatgacaccgac	20	MLOC_70788.1	800	–	c:159(2.18|1)+	2	–35.5
miR1432–5p	tcaggagagatgacaccgac	20	MLOC_70272.1	1299	–	c:295(7.20|1)+	4	–39.6
miR1432–5p	tcaggagagatgacaccgac	20	MLOC_61497.1	1339	c:1005(14.12|3)*	c:1006(54.61|1)+	5	–30.2
miR6201–5p	tgaccctgaggcactcataccg	22	MLOC_52074.1	653	c:209(2.01|10)*	c:210(2.01|10)+	13.5	–26.2

barley stages (except for 5'U-miR156-5p in 2-week old barley), when compared to the 1-week stage (Fig. 1A–H, Suppl. Table 1). MiR168-3p was earlier considered as a passenger miRNA for miR168-5p. MiR168-3p has a higher abundance level at all stages when compared to the 1-week-old barley plants, with the highest 2.8 fold change in 2-week-

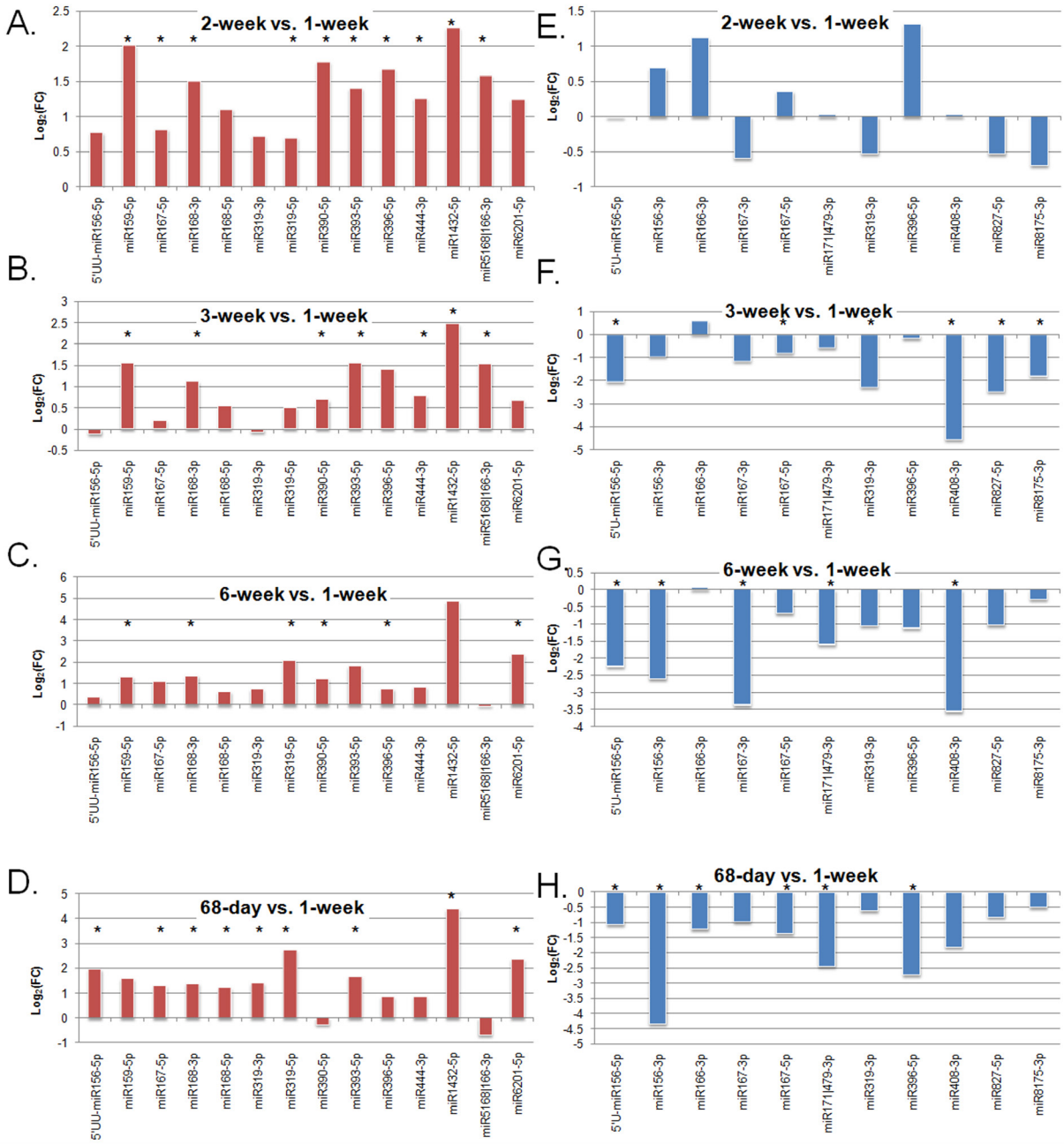

Figure 1. Fold change of microRNA expression in:
(**A**) 2- *vs.* 1-week-old barley; (**B**) 3- *vs.* 1-week-old barley; (**C**) 6- *vs.* 1-week-old barley; (**D**) 68-day- *vs.* 1-week-old barley; (**E**) 2- *vs.* 1-week-old barley; (**F**) 3- *vs.* 1-week-old barley; (**G**) 6- *vs.* 1-week-old barley; and (**H**) 68-day- *vs.* 1-week-old barley. Red and blue bars represent the elevated and decreased microRNA levels, respectively. Fold changes a(re represented as $\log_2$ (FC) values. Symbol * depicts *p*-value <0.05. Detailed information about presented microRNA is included in Suppl. Table 1.

old plants (Fig. 1A–D). The degradome-approved mRNA target for this miRNA encodes the regulator of chromosome condensation (RCC1) protein (Table 2).

The level of miR168-5p (21 nt) was only significantly increased in 68-day-old barley plants (Fig. 1A–D). Arabidopsis miR168-5p targets *Ago1* mRNA; thus, it controls the post-transcriptional gene silencing process. We also found miR168-5p guided *Ago1* mRNA cleavage fragments in the barley degradome data. (Table 1, Table 2).

MiR1432-5p levels highly increased in 6-week- and 68-day-old barley, which targeted mRNAs encoding protein possessing EF-hand, calcium binding motif, and protein with 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase domain (Figs. 1C–D; Fig. 2B; Table 1, Table 2, Suppl. Table 2 at www.actabp.pl). The obtained pre-miR1432 structure shows the presence of a properly folded stem-loop (Fig. 3A).

MiR156-5p is a highly abundant microRNA in all barley developmental stages. In rice, there are 12 miR156-5p species (a-l), and all of them target *SPL* TF mRNAs. The most abundant 20 nt 5'U-miR156-5p (5' TGACAGAAGAGAGTGAGCAC 3') molecule is present in 1- and 2-week-old barley. In older stages, the level of this microRNA is much lower (Figs. 1F–H, Fig. 2D). The target mRNAs identified in the barley degradome data encode six TFs (Table 1, Table 2). MiR159-5p expression was significantly

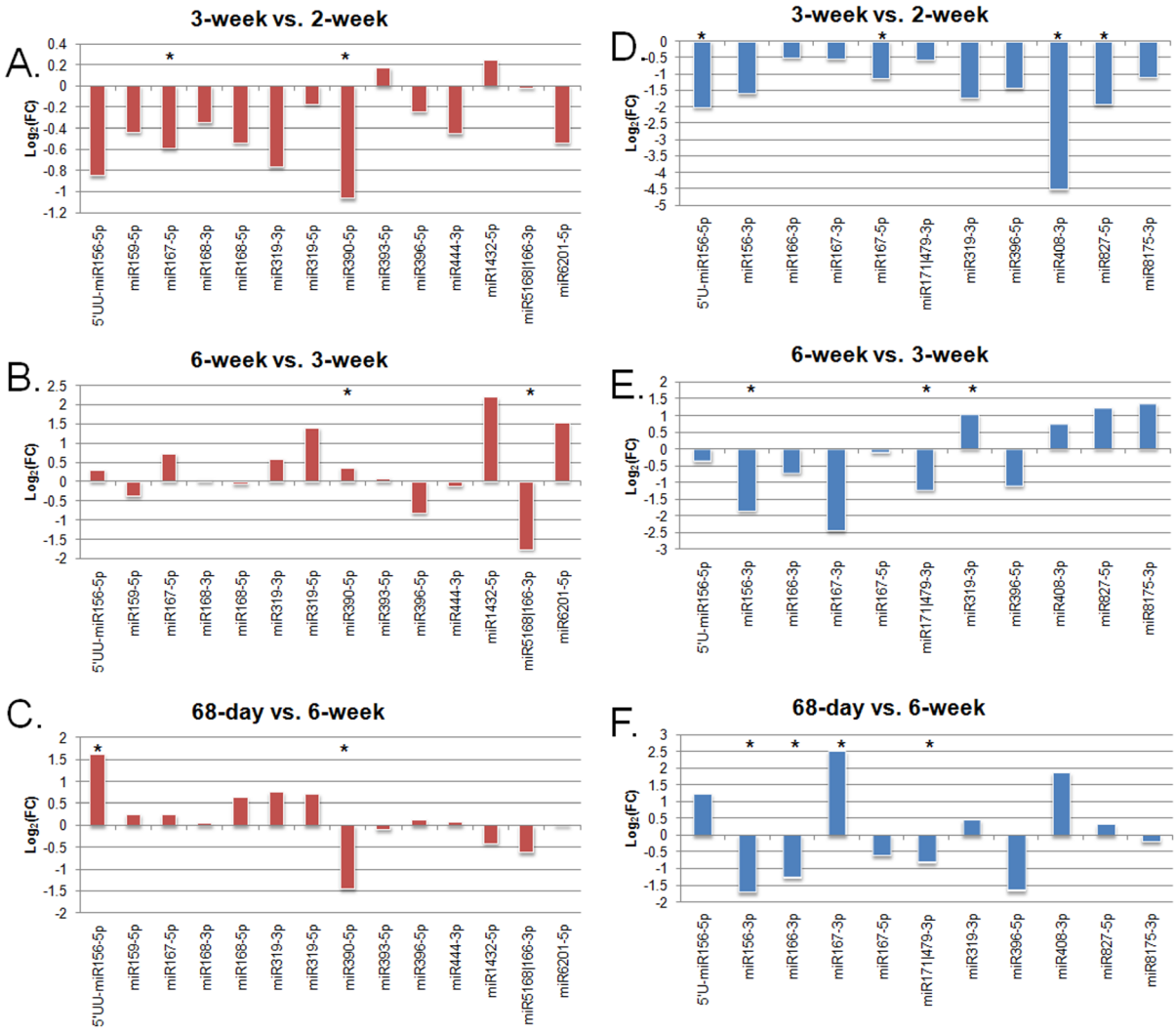

Figure 2. Fold change of microRNAs expression in:
(**A**) 3- *vs*. 2-week-old barley; (**B**) 6- *vs*. 3-week-old barley; (**C**) 68-day- *vs*. 6-week-old barley; (**D**) 3- *vs*. 2-week-old barley; (**E**) 6- *vs*. 3-week-old barley; and (**F**) 68-day- *vs*. 6-week-old barley. Red and blue bars on the charts represent the same set of microRNA molecules which were analyzed in Fig. 1. Symbol * depicts *p*-value <0.05.

higher in 2-, 3-, and 6-week-old barley plants; however, in 68-day-old barley, its expression increased by $\log_2(FC)=1.58$, but not significantly (Figs. 1A–D, Suppl. Table 1 at www.actabp.pl). The degradome data identified its mRNA target encoding pyruvate decarboxylase (Table 1, Table 2). However, in the 68-day developmental stage, we observed an increased level of the miR159-3p isomiR (Suppl. Table 1 at www.actabp.pl). In the degradome data we found target for this miRNA; i.e., an mRNA encoding LIM-domain binding protein (Table 2). In 68-day-old barley, we identified another miR159-3p isomiR that is more abundant than the previous one, and it was expressed higher in the 68-day-old barley than in 1-week-old barley (but not significantly). With the degradome data, we identified its target mRNA – MYB TF (Table 1, Table 2).

MicroRNA expression level as a specific marker for a given developmental stage

Comparison of microRNA expression in 2- *versus* 1-week-old barley plants.

It is known that leaves and new tillers start to develop in the 2-week-old barley plants. At this developmental stage, the level of the following eleven microRNAs is significantly increased: miR156-5p, miR159-5p, miR168-5p/-3p, miR172-5p, miR390-5p, miR393-5p, miR396-5p, miR444-3p, miR528-3p, miR1432-5p, and miR5168-3p (Fig. 1A, Suppl. Table 1 at www.actabp.pl). Some of these microRNAs are directly related to the barley development. For reference, miR393-5p targets mRNAs that encode Transport Inhibitor Response1 (TIR1) and Auxin Signaling F box 2 and 3 proteins (AFB 2, AFB3) involved in the leaf development (Si-Ammour *et al.*, 2011). MiR396 targets *GRF1* (Growth-Regulating Factor 1) and *GRF9* mRNA (Wang *et al.*, 2011). In Arabidopsis, AtGRF1 TF is required for the coordination of cell division and differentiation during leaf development (Wang *et al.*, 2011). Since miR396-5p is the most-abundant microRNA (Fig. 1A, Suppl. Table 1 at www.actabp.pl), this may suggest the elevated importance of miR396-5p at the 2-week-old barley developmental stage. With the degradome data, we identified their mRNA targets as *TIR1*, *AFB*, and *GRF* (Table 1, Table 2). As mentioned earlier miR159-3p targets MYB TF mRNA (Allen *et al.*, 2010). We observed that miR159-5p is at a higher level in the 2-week-old barely (Fig. 1A). Degradome analysis revealed target mRNA encoding pyruvate decarboxylase

Table 2. Identification of the predicted target.
Accession number – Ensembl Plants database, GenBank database.

microRNA	Accession number	Protein function
miR156-5p	MLOC_61297.1	Squamosa promoter-binding-like protein, BAJ94319.1
miR156-5p	MLOC_37841.5	Squamosa promoter-binding-like protein, BAK05794.1
miR156-5p	MLOC_52321.1	Squamosa promoter-binding-like protein, BAJ87295.1
miR156-5p	MLOC_11199.7	Squamosa promoter-binding-like protein, BAJ97637.1
miR156-5p	MLOC_13032.1	Squamosa promoter-binding-like protein
miR156-5p	MLOC_62426.1	Squamosa promoter-binding-like protein, BAJ92814
miR159-5p	MLOC_38762.5	Pyruvate decarboxylase
miR159-3p	MLOC_55324.1	uncharacterized protein
miR159-3p	MLOC_71332.2	MYB TF
miR159-3p	MLOC_72581.1	TCP TF
miR159-3p	MLOC_75369.1	LIM-domain binding protein
miR167-5p	MLOC_58330.5	Auxin response factor (ARF)
miR168-5p	MLOC_12447.2	Argonaute 1
miR168-3p	MLOC_63404.6	Regulator of chromosome condensation (RCC1) family protein
miR169-3p	MLOC_63790.1	Serine/Threonine-protein kinase
miR171-5p	MLOC_11773.1	Alpha, alpha-trehalose-phosphate synthase (UDP-forming)
miR319-5p	MLOC_61362.3	CBL-interacting protein kinase 23
miR319-3p	MLOC_71332.2	MYB TF
miR390-5p	MLOC_37712.1	GRAS (GAI, RGA, SCR) protein family, chitin-inducible gibberellin-responsive protein 1
miR390-5p	MLOC_33978.3	Homeobox-leucine zipper protein HOX32
miR393-5p	MLOC_56088.1	Auxin Signaling F-BOX
miR393-5p	MLOC_9864.2	Transport Inhibitor Response 1
miR396-5p	MLOC_80060.1	Growth-Regulating Factor 9, GRF9
miR396-5p	MLOC_67201.2	Growth-Regulating Factor 2
miR408-3p	MLOC_69288.1	Blue copper protein
miR827-5p	MLOC_52144.1	RNA helicase
miR827-5p	MLOC_70149.1	SRG1 protein
miR827-5p	MLOC_59423.5	protein with DUF1668 domain
miR1432-5p	MLOC_70272.1	EF-hand, calcium binding motif
miR1432-5p	MLOC_70788.1	EF-hand, calcium binding motif
miR1432-5p	MLOC_61497.1	Naringenin, 2-oxoglutarate 3-dioxygenase, 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
miR6201-5p	MLOC_52074.1	66 AA protein with uncharacterized function

for this miRNA (Table 1, Table 2). MiR528-3p has the second highest up-regulation level (8.6 fold change in comparison to 1-week-old plants) of all of the up-regulated miRNAs at this developmental stage (Suppl. Table 1 at www.actabp.pl). We also identified and folded its pre-miR stem-loop structure (Fig. 3B). Since we did not identify any target for this miRNA in the degradome data, we used psRNATarget software to predict a putative one (Dai & Zhao, 2011). We identified two potential targets of the miR528-3p, described as Caleosin 1 (Clo1, acc BI949541, translational inhibition) and aconitate hydratase mRNA (acc BI952160, mRNA cleavage).

Comparison of microRNA expression in 3- *versus* 1-week-old barley plants.

A 3-week-old barley plant develops new tillers. Among eight significantly up-regulated microRNAs, the highest expression in the 3-week old barley was detected for miR168-3p, miR159-5p and miR393-5p (Fig. 1B, Suppl. Table 1 at www.actabp.pl). Eight microRNA species have significantly lower levels when compared to the 1-week-old barley plants: miR156-5p/-3p, miR167-5p, miR169-5p/-3p, miR319-3p, miR408-5p/-3p, miR827-5p, miR5072-5p (16 nt) and miR8175-3p (Suppl. Table 1 at www.actabp.pl). In the cases of miR156-5p and miR408-3p, we observed a rapid down-regulation of their levels. The degradome analysis points to the mRNA target for miR408-3p that encodes the Blue copper protein mRNA (Table 1, Table 2). MiR167-5p was up-regulated in the 3-week-old barley plants (Suppl. Table 1 at www.actabp.pl). Interestingly, we found the highest level of canonical 21-nt-long miR167-5p in the 3-week-old barley plants; however, two different 22-nt-long miR167-5p isomiRs have an even higher abundance

Table 3. The average normalized reads of miR167-5p in five barley developmental stages.

miR167-5p	length, bp	1-week	2-week	3-week	6-week	68-day
tgaagctgccagcatgatctg	21	45	81	121	93	106
tgaagctgccagcatgatctgc	22	3011	6462	6191	4349	3415
tgaagctgccagcatgatctga	22	1718	3243	2112	3990	4231
tgaagctgccagcatgatctgg	22	10	23	15	22	31

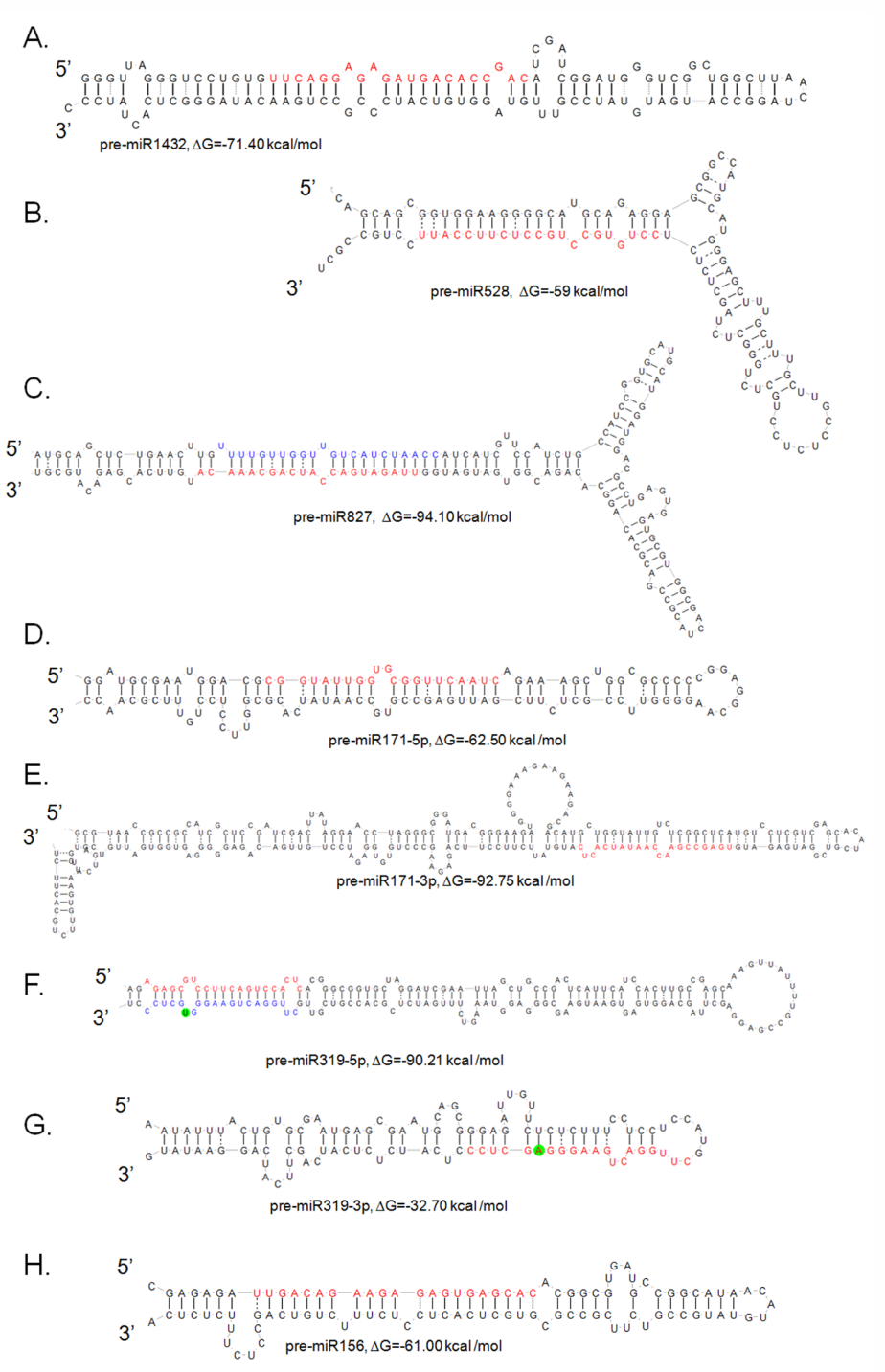

Figure 3. Stem-loop structures of the selected barley pre-miRNAs:
(**A**) pre-miR1432; (**B**) pre-miR528; (**C**) pre-miR827; (**D**) pre-miR171-5p; (**E**) pre-miR171-3p; (**F**) pre-miR-319-5p; (**G**) pre-miR319-3p; and (**H**) pre-miR156 for 5′UU-miR156-5p. Red and blue letters represent microRNA or microRNA*/non-canonical microRNA sequence, respectively. Structures with the lowest free energy are presented.

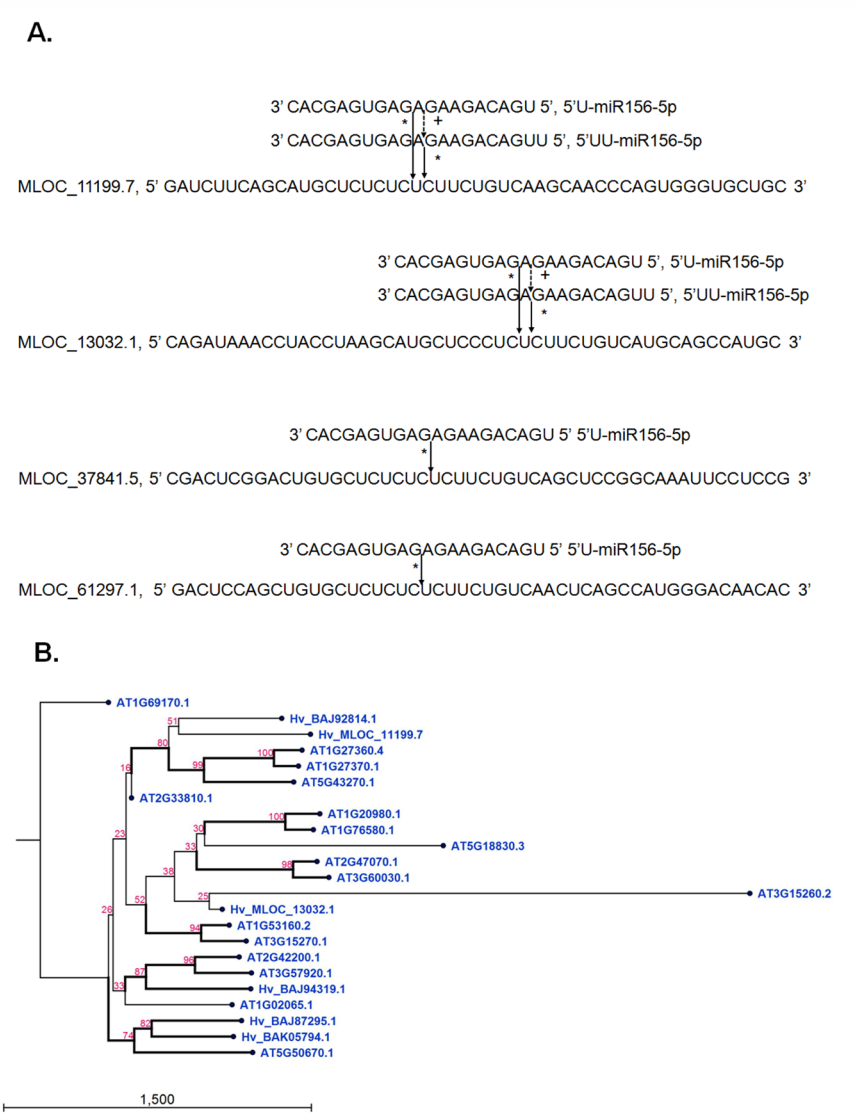

Figure 4. (A) The cleavage sites in MLOC_11199.7, MLOC_13032.1, MLOC_37841.5 and MLOC_61297.1 targeted by 5′UU-miR156-5p and/or 5′U-miR156-5p. (B) Phenogram of Arabidopsis SPL TF amino-acids sequences and six barley SPL TFs.
Barley MLOC_11199.7, MLOC_13032.1, MLOC_52321.1, MLOC_62426.1 mRNA are targeted by 5′UU-miR156-5p and 5′U-miR156-5p; MLOC_37841.5, MLOC_61297.1 mRNA are targeted exclusively by 5′ U-miR156-5p. The tree was constructed using N-J algorithm, bootstrap = 1000 replication, CLC Main Workbench (Qiagen) software. *Arabidopsis thaliana* SPL TFs accession numbers: AT2G47070.1 – SPL1; AT5G43270.1 – SPL2; AT2G33810.1 – SPL3; AT1G53160.2 – SPL4, AT3G15270.1 – SPL5, AT1G69170.1 – SPL6; AT5G18830.3 – SPL7; AT1G02065 – SPL8; AT2G42200.1 – SPL9; AT1G27370.1 – SPL10; AT1G27360 – SPL11; AT3G60030.1 – SPL12; AT5G50670.1 – SPL13B; AT1G20980.1 – SPL14; AT3G57920.1 – SPL15; AT1G76580.1 – SPL16, Barley SPL TF accession numbers: MLOC_11199.7, MLOC_13032.1, BAJ87295 (MLOC_52321.1), BAJ92814 (MLOC_62426.1), BAK05794 (MLOC_37841.5), BAJ94319 (MLOC_61297.1).

(Table 3). MiR167-5p targets *ARF6* and *ARF8* (Auxin Response Factor) mRNAs (Liu *et al.*, 2014) and in barley also *NEK5-like kinase* (Kruszka *et al.*, 2014). In the degradome data, we also found *ARF* mRNA targeted by miR167-5p. MiR827-3p expression is induced by phosphate starvation (Hackenberg *et al.*, 2013). In Arabidopsis, this microRNA targets *NLA* mRNA (*Nitrogen Limitation Associated*) that encodes the E3 ubiquitin ligase involved in Pi-related protein degradation *via* the proteasome pathway. In rice and barley, miR827-3p targets the SPX-MFS mRNA-encoding protein involved in Pi sensing and transport (Lin *et al.*, 2010; Hackenberg *et al.*, 2013). Interestingly, we also identified miR827-5p that was expressed at a lower level in the 3-week-old barley plants (Fig. 1F). MiR827-5p and miR827-3p are derived from the same precursor (Fig. 3C). Based on the degradome analysis, we identified several mRNA targets that are potentially targeted by miR827-5p (Table 1, Table 2).

Comparison of microRNA expression in 6- *versus* 1-week-old barley plants.

The 6-week-old barley plant elongates its shoots and is still before its flowering time. The highest expression levels were recorded for the following microRNAs: miR168-3p, miR159-5p, miR319-5p, miR6201-5p, miR390-5p, and 20 nt miR1432-5p isomiR (Fig. 1C, Suppl. Table 1 at www.actabp.pl). Interestingly the highest abundance of 20 nt and 21 nt miR1432-5p isomiRs is specific for this stage (Suppl. Table 1 at www.actabp.pl). Moreover, up-regulation of miR390-5p is observed (Fig. 1C, Fig. 2B, 2C), although the highest miR390-5p level fold change is observed for 2-week *vs.* 1-week-old

Table 4. MiR156 with significant expression fold change in 68-day- *vs*. 1-week-old barley.

miR	sequence	average reads no (68-day old)	log_2(FC)	*p*-value
5′UU-miR156-5p	ttgacagaagagagtgagcac	12749.3525	1.96	0.001116
5′U-miR156-5p	tgacagaagagagtgagcac	10157.55	–1.07	0.022749
miR156-5p	tgacagaagagagtgagtac	8.8475	–2.7	0.045473
miR156-5p	acagaagagagtgagcaca	0.565	–2.99	0.027137
miR156-5p	tgacagaagagagtgagcaca	16.9375	–2.92	0.036196
miR156-3p	gctcactgctctatctgtcacc	5.04	–4.35	0.004855

barley (Fig. 1A). It is known that Arabidopsis miR390-5p is recruited by an AGO7 and targets *TAS3* RNA (Fahlgren *et al*., 2006). As a consequence, ta-siRNAs are produced. In the subsequent steps, ta-siRNAs target *ARF-2*, *ARF-3*, and *ARF-4* mRNAs, which control lateral root growth (Marin *et al*., 2010; Endo *et al*., 2013). We did not find a barley *TAS3* homologue in either the Ensemble Plants or psRNATarget databases. However, the barley degradome analysis allowed us to find other genes, described as MLOC_37712.1 – belonging to the GRAS (GAI, RGA, SCR) protein family, chitin-inducible gibberellin-responsive protein 1, and MLOC_33978.3 encoding Homeobox-leucine zipper protein (HOX32), as targets for miR390-5p. The highly expressed miR1432-5 targets the mRNAs that encode the 2-oxoglutarate 3-dioxygenase protein as well as the protein with EF-hand, calcium-binding motifs (Table 1, Table 2).

Two microRNAs (miR171-5p and miR319-5p) exhibit elevated expression in the 6-week-old barley plants; however, their counterparts (miR171-3p and miR319-3p) show concomitantly lower expression levels (Suppl. Table 1 at www.actbp.pl). Rice miR171c-3p targets four *GRAS* (GAI-RGA-SCR) plant-specific transcription factors mRNA (Fan *et al*., 2015). PsRNATarget analyses revealed another mRNA target for the barley miR171-3p encoding the LRR receptor-like serine/threonine-protein kinase (TC239462). The target sequence for the miR319-5p was identified as CBL-interacting protein kinase 23 mRNA, for the 20-nt-long miR319-3p the mRNA target encodes the MYB TF (Table 1, Table 2). The miR319-5p is 21 nt in length, is expressed at a high level, and is more abundant than the 20-nt miR319-3p that is down-regulated. Interestingly, we found that mentioned above miR171-5p/miR171-3p, and miR319-5p/miR319-3p are not derived from the same precursors (Fig. 3D–G).

Comparison of microRNA expression in 68-day- *versus* 1-week-old barley plants.

The 68-day-old barley plants have already developed spikes with kernels at the milk stage. Thirteen microRNAs were identified as having significantly higher levels; among them are: miR156-5p (5′UU – 21 nt), miR168-5p, miR319-3p, miR167-5p, miR393-5p, miR1432-5p, miR6201-5p. In contrast to all of the earlier developmental stages, the isomiR 5′UU-miR156-5p exhibited a uniquely higher level when compared to the 1-week-old plants (Fig. 1D). This isomiR is identical to *Glycine max* miR156k/n/o isomiRs and can be diced out from the pre-miR156 precursor (Fig. 3H). On the other hand, the 5′U-miR156-5p was down-regulated (Fig. 1H, Table 4). We analysed six *SPL* mRNA targets of the miR156-5p isomiRs and found that the four mRNA targets can be potentially recognized by both, the 5′UU- and 5′U-miR156-5p (21 nt and 20 nt) isomiRs, but two other targets can be recognized exclusively by the 5′U-miR156-5p (Fig. 4A, Table 1, Table 2). Moreover, comparison with the known Arabidopsis SPL TFs showed that the barley targets belong to a different SPL TF class (Fig. 4B). SPL TF encoded by the MLOC_612971 was targeted by the 5′ U-miR156-5p and is similar to Arabidopsis SPL9 and SPL15 TFs.

MiR1432-5p, miR6201-5p are monocot specific (Schreiber *et al*., 2011; Pandey *et al*., 2014). They are especially expressed in the 6-week- and 68-day-old barley plants. MiR6201 was predicted to have one mRNA target in wheat (Pandey *et al*., 2014), encoding a protein similar to proteinase inhibitor Rgpi9 (Pandey *et al*., 2014). In the degradome data, we found one target (MLOC_52074.1) that encodes a short 66 AA peptide of unknown function.

DISCUSSION

Deep sequencing of small RNAs is a powerful tool to discriminate between microRNA sequences and their abundance (Sobkowiak *et al*., 2012; Lukasik *et al*., 2013; Barciszewska-Pacak *et al*., 2015). Analysis of the changes in small RNA level between the particular developmental stages of barley plants revealed some interesting data, of which two observations seem to be the most profound: (i) the 20-nt 5′U-miR156-5p level decreases during barley development, while in spike (68-day-old plants), upregulation of the 21-nt 5′UU-miR156-5p is observed. Both isomiRs target the same mRNAs, but the shorter one additionally guides the cleavage of two unique mRNAs; (ii) the level of miR390-5p was specifically elevated only in the 2-week and 6-week-old plants (more than 2 fold). Some interesting observations concern the microRNA and its microRNA* levels. Previously, we identified miR159b-3p as an up-regulated microRNA during barley development (Kruszka *et al*., 2013). Here, we found that, apart from the miR159b-3p increase, the highest fold change is observed for its microRNA* – miR159b-5p. MiR168-3p is up-regulated at all stages as compared to the 1-week-old barley, while the 21 nt miR168-5p is significantly up-regulated only in the 68-day-old barley plants. Our previous work had shown that the level of miR168-5p was almost unchanged and the level of miR168-3p was even decreased when compared to the 1-week barley stage (Kruszka *et al*., 2013). However, the previous analysis was based on northern blot hybridization, which is less sensitive and often does not discriminate between microRNAs belonging to the same family. Thus, sRNA deep sequencing enables a much-deeper analysis of microRNAs derived from the same family or isomiRs.

It is known that the miR156-5p expression profile is the opposite of the miR172 expression pattern. In rice, miR156 targets 11 *SPL* genes (Xie *et al*., 2006; Xie *et al*., 2012). SPL TFs regulate miR172 expression (Spanudakis & Jackson, 2014). It is possible that the same regulation occurs in barley plants. In further studies, it will be interesting to establish which miR156-5p isomiR affects the miR172 expression in barley. As previously mentioned, 5′U-miR156-5p 20 nt is down-regulated, but its 21-nt isomiR with additional U residue at the 5′ end is

up-regulated in the 68-day-old barley when compared to 1-week-old plants. One additional nucleotide at the 5'end of the miR156-5p allows for the recognition of different *SPL* mRNAs. In the degradome dataset, we found indeed that different *SPL* mRNAs are targeted by these two isomiRs of the miR156-5p. Comparison with known Arabidopsis SPL TFs showed that *SPL* mRNA that are targets for miR156-5p belong to different SPL classes. In agreement with these observations, we found that 20-nt 5'U-miR156-5p recognizes *SPL* mRNAs similar to Arabidopsis SPL9 and SPL15 TF mRNAs (Fig. 4B). Thus, microRNA isomiRs derived from a single pri-miRNA can specifically regulate various *SPL* gene expression. It will be interesting to find an exact relationship between the up- and down-regulated miR156-5p isomiRs and their *SPL* mRNA targets. The deep-sequencing data of sRNAs isolated from plants from several developmental stages confirm earlier observations published in our previous paper (Kruszka *et al*., 2013): Northern blot hybridization showed the presence of two microRNAs156-5p that differed in size. The northern signals in the 68-day-old barley reflect up-regulated 21-nt-long 5'UU-miR156-5p. In the mirEX2.0 database, there are expression profiles of three barley miR156 family members: miR156b, miR156c, and miR156d. These three microRNA156a/b/c are downregulated in the 6-week- and 68-day-old barley plants. This reflects the decreased expression of 5'U-miR156-5p. MiR156 expression decreased in shoots or whole rice plants; however, careful inspection of selected organs revealed that this microRNA level is increased in older leaves (Xie *et al*., 2012). The authors did not discriminate between the isomiRs. Here, we have shown that, in barley plants at the spike-formation stage (68-day-old plants), there are two miR156-5p izomiRs, which are differentially expressed.

Compared to the 6-week-old plants, we observed a decreased level of miR390-5p in the 68-day-old barley plants. This microRNA regulates the production of Arabidopsis ta-siRNAs that, in turn, downregulate ARF2, ARF3, and ARF4 TF levels. It was shown that the decreased level of ARF2, ARF3, and ARF4 TFs promotes lateral root growth (Marin *et al*., 2010). It would be interesting to test the level of these transcription factors in the 68-day-old barley plants and during lateral root growth. One of the elevated microRNAs in older barley plants is the miR1432-5p that targets the mRNA-encoding protein containing 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase domains. We blasted the barley MLOC_61497.1 (target of the miR1432-5p) to the NCBI protein database. The highest similarity to this barley sequence was found in the case of Naringenin, 2-oxoglutarate 3-dioxygenase from *Triticum urartu* (EMS57783.1, E value=0.0, identities=95%, 323/339). This enzyme catalyzes the 3-beta-hydroxylation of 2S-flavanones to 2R, 3R-dihydroflavonols, which are intermediates in the biosynthesis of flavonols, anthocyanidins, catechins, and proanthocyanidins. Altogether, we observed that during barley development, microRNA expression profiles change qualitatively and quantitatively. These changes influence the mRNA target levels and plant development.

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