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RESEARCH ARTICLE

Genome-wide analysis of brassinosteroid responsive small RNAs in *Arabidopsis thaliana*

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Abstract

Background Brassinosteroids (BRs) are a class of phytohormones with important roles in regulating physiological and developmental processes. Small RNAs, including small interfering RNAs and microRNAs (miRNAs), are non-protein coding RNAs that regulate gene expression at the transcriptional and post-transcriptional levels. However, the roles of small RNAs in BR response have not been studied well.

Objective In this study, we aimed to identify BR-responsive small RNA clusters and miRNAs in Arabidopsis. In addition, the effect of BR-responsive small RNAs on their transcripts and target genes were examined.

Methods Small RNA libraries were constructed from control and epibrassinolide-treated seedlings expressing wild-type BRI1-Flag protein under its native promoter in the *bri1-5* mutant. After sequencing the small RNA libraries, differentially expressed small RNA clusters were identified by examining the expression levels of small RNAs in 100-nt bins of the Arabidopsis genome. To identify the BR-responsive miRNAs, the expression levels of all the annotated mature miRNAs, registered in miRBase, were analyzed. Previously published RNA-seq data were utilized to monitor the BR-responsive expression patterns of differentially expressed small RNA clusters and miRNA target genes.

Results In results, 38 BR-responsive small RNA clusters, including 30 down-regulated and eight up-regulated clusters, were identified. These differentially expressed small RNA clusters were from miRNA loci, transposons, protein-coding genes, pseudogenes and others. Of these, a transgene, *BRI1*, accumulates small RNAs, which are not found in the wild type. Small RNAs in this transgene are up-regulated by BRs while *BRI1* mRNA is down-regulated by BRs. By analyzing the expression patterns of mature miRNAs, we have identified BR-repressed miR398a-5p and BR-induced miR156g. Although miR398a-5p is down-regulated by BRs, its predicted targets were not responsive to BRs. However, *SPL3*, a target of BR-inducible miR156g, is down-regulated by BRs.

Conclusion BR-responsive small RNAs and miRNAs identified in this study will provide an insight into the role of small RNAs in BR responses in plants. Especially, we suggest that miR156g/SPL3 module might play a role in BR-mediated growth and development in Arabidopsis.

Keywords Brassinosteroid · Small RNA · MicroRNA · Arabidopsis thaliana

So Young Park and Jae-Han Choi contributed equally to the work.

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Introduction

Brassinosteroids (BRs), as phytohormones, are essential for plant growth and development including biotic and abiotic stresses tolerance in higher plants (Oh et al. 2020). Most of BR signaling research focused on plant growth and development with identifying essential signaling components from the plasma membrane to nucleus. Mutants for either BR biosynthesis or normal signaling pathways share similar developmental defects including severe dwarfism, rounded leaves, shortened petioles, flowering time, senescence, reduced male fertility, and other abnormal photomorphogenesis (Clouse et al. 1996; Friedrichsen et al. 2000; Li and Chory 1997; Noguchi et al. 1999). As the first BR signaling mutant, *bri1 brassinosteroid insensitive 1* (*bri1*) with EMS treatment of Arabidopsis seeds, was identified through a loss of function genetic screen for BR insensitivity with root growth in the presence of BR (Clouse et al. 1996). BRs are perceived by the plasma membrane-localized and leucine-rich repeat (LRR) receptor kinase BRI1 (Wang et al. 2001). In the absence of BRs, BRI1 KINASE INHIBITOR 1 (BKI1) binds to BRI1 to prevent heterodimerization between BRI1 and its co-receptor, BRI1 ASSOCIATED KINASE 1 (BAK1) (Li et al. 2002; Nam and Li 2002). GLYCOGEN SYNTHASE KINASE 3 (GSK3)- like kinase,BRASSINOSTEROID INSENSITIVE 2 (BIN2), phosphorylates bri1 EMS SUPPRESSOR1/BRASSINAZOLE RESISTANT1 (BES1/BZR1) family transcription factors (He et al. 2002). BRI1 encodes a serine/threonine (Ser/Thr) leucine-rich repeat receptor-like protein kinase (LRR-RLK) (Li and Chory 1997). Biochemical analysis indicated BRI1 is a critical component for BR perception and signal transduction (Wang et al. 2001). Genetic and biochemical analyses strongly support that heterodimerization of BRI1 and BAK1 may be important for BR signaling (Li et al. 2002; Nam and Li 2002). In the presence of BR, BRI1 receptor perceived BR on island domain of extracellular region and eventually, BKI1 is phosphorylated and disassociated from BRI1 (Jaillais et al. 2011; Wang and Chory 2006), which leads to the association of BRI1 with co-receptor BAK1 (Bucherl et al. 2013; Sun et al. 2013). After heterodimerization of both receptor kinases, autophosphorylation and transphosphorylation between BRI1 and BAK1 then lead to the activation of BRI1 kinase (Wang et al. 2008) and serial signaling cascaded to downstream through phosphorylation and activation of bri1 SUPPRESSOR 1 (BSU1), a Kelch-repeat domain-containing protein phosphatase, leading to the dephosphorylation and inactivation of BIN2 (Ryu et al. 2007; Wang et al. 2002). The inhibition of BIN2 and action of PROTEIN PHOSPHOTASE 2A (PP2A) promotes the nuclear accumulation of non-phosphorylated BES1/BZR1 (Di Rubbo et al. 2011; Tang et al. 2011). As likely master transcription factor, BES1/BZR1 interconnected to many other transcriptional regulators to control the expression of thousands of genes.

Plants have to deal with a wide array of signals generated by an environment. Many signals from the environment, such as light, temperature, and pathogens, influence how and when a plant decides to grow (Vert and Chory 2011). Many scientists tried to identify diverse BR- response genes in higher plants through employing microarray analysis or RNA sequencing techniques. In all multicellular organisms, growth must be synchronized, but for plants, this is of particular importance because the appropriate timing of growth occurs in response to specific signals. Thus, the coherence and the time structure of plant growth not only require mechanisms to infer the current and future state of the growth condition but also need to be connected to rapid and robust regulation mechanism systems. Structurally diverse small- molecule, phytohormones, ABA, auxin, GA, BR, cytokinin, ethylene, and strigolactone) assists plants in responding appropriately to the environment (Finet and Jaillais 2012). Although diverse plant hormones are thought to form the central frameworks of plant growth and development, the growth-promoting molecules influence each other by reprogramming the respective structures of their signaling backbone. The program of plant growth is coordinated temporally and spatially by an intricate network of molecular regulators in the cells.

Recent studies revealed that non-coding small RNAs play critical roles in plant development and stress responses via phytohormone signaling pathways. In this study, we focus on the microRNAs (miRNAs) involved in BR signaling pathway. The first miRNA was discovered in the nematode *Caenorhabditis elegans* (Lee et al. 1993). miRNAs were classified as a separate distinct class of RNAs (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). Currently, 8604 mature miRNAs and 6882 precursor miRNAs (pre-miRNAs) have been identified in 73 plant species (Kozomara and Griffiths-Jones 2014). As we know, miRNAs are non-coding RNA molecules which are 19–24 nt in length and function as gene regulators in diverse organisms. In higher plants, miRNA regulate diverse biological processes including organ development, phase transition including flowering (Chuck et al. 2009; Damodharan et al. 2016; Kamthan et al. 2015; Li and Zhang 2016; Meng et al. 2010; Nova-Franco et al. 2015; Rubio-Somoza and Weigel 2011) abiotic and biotic stress tolerance (Hackenberg et al. 2015; Jeong et al. 2011; Karimi et al. 2016; Li et al. 2011; Naya et al. 2014; Niu et al. 2016; Stief et al. 2014; Sunkar and Zhu 2004; Wang et al. 2011; Xie et al. 2015). Since the discovery of the first miRNA, a wide range of studies has provided clear evidence for the involvement of miRNAs in many biological processes including stress responses.

Interestingly, we already know, BR mediated abiotic and biotic stresses tolerance in plants even though the mechanisms are still unclear in higher plants (ref). Therefore, we performed miRNA analysis using Arabidopsis seedling to identify specific mRNAs, which are regulated by BR.

Materials and methods

Plant material and growth conditions

Plants expressing wild-type BRI1-Flag protein under its native promoter in the *bri1-5* mutant were used at the plant material. Seeds were surface sterilized, kept at 4 °C for 2 days, and *Arabidopsis thaliana* plants were grown in shaking liquid culture as previously described (Oh et al. 2009) and plants were treated with 1 μM epibrassinolide (epiBL) or solvent control for 90 min before harvest.

Small RNA library construction and sequencing

Total RNAs, including small RNA fractions, were isolated using mirVana™ miRNA isolation kit (ThermoFisher, Waltham, MA) from Arabidopsis seedlings treated with mock or epiBL for 90 min, with three biological replicates. Small RNA library was prepared using the TruSeq small RNA sample preparation protocol (Illumina, San Diego, CA), barcoded, and sequenced on an Illumina GAxII platform.

Small RNA cluster analysis

Small RNA reads were adaptor-trimmed and mapped to the Arabidopsis genome (TAIR10) sequence using bowtie2 (Langmead and Salzberg 2012). Reads unambiguously mapped to TAIR10 features, including known miRNA loci, were counted using a custom python script. Adaptor-trimmed reads of length 21–24 nt aligned to the 100-nt bins in each strand of the Arabidopsis genome sequences were counted. The starting position of each alignment was considered when assigning reads to the 100-nt bins. DESeq (Anders and Huber 2010) was used to identify 100-nt bins showing significantly different expression between control and epiBL-treated samples (adjusted p value < 0.05) among all 100-nt bins with on average more than 5 reads aligned to them across the samples. Integrative Genomics Viewer (Robinson et al. 2011) was used to visually confirm the differential expression patterns.

Analysis of differentially expressed miRNA

miRNA expression analysis was conducted using CLC genome workbench small RNA pipeline (Qiagen, Denmark). After pre-processing, miRNA reads were retrieved by mapping the small RNA reads to the know Arabidopsis miRNAs from miRBase Release 22.1 (Kozomara and Griffiths-Jones 2014). After normalization of miRNA reads with TP4M (Transcripts Per 4 Million reads), differentially expressed miRNAs were identified by a twofold difference between control and epiBL-treated seedlings with statistic significance of p value < 0.001.

RNA-seq analysis of miRNA targets

The experimentally validated target genes for miR156g and miR398a-3p were obtained from our previous data (Jeong et al. 2013a). Putative targets for miR398a-5p were computationally predicted using psRNATarget program (Dai and Zhao 2011). The expression patterns of these miRNA targets were analyzed using RNA-seq data. Previously published RNA-seq data (Oh et al. 2014) of control and epiBL-treated Arabidopsis seedlings were downloaded from National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) under accession number GEO GSE51772 (GSM1252262, GSM1252263, GSM1252264, GSM1252265). RNA-seq reads were processed and mapped to Arabidopsis genome (TAIR10) sequence using CLC genome workbench RNA-seq pipeline (Qiagen, Denmark). The expression level of each gene was normalized as RPKM (Reads Per Kilobase of transcripts per million reads) and differentially expressed genes were analyzed according to the manufacturer's instructions.

Results and discussion

Profiling of small RNAs from epibrassinolide-treated and mock-treated Arabidopsis seedlings

To investigate the role of small RNAs in BR responses in Arabidopsis, three biological replicates of small RNA libraries were constructed and sequenced from epiBL-treated and mock-treated control seedlings for 90 min. Sequencing results showed that approximately four million raw reads per library were obtained. After processing of adapter sequences and filtering out low-quality sequence reads, clean small RNAs were mapped to the Arabidopsis genome sequences. All mapped sequences were classified according to non-coding RNA gene, protein-coding gene, pseudogene, transposable element gene, and intergenic regions (Table 1). Of non-coding RNA genes, the majority of 21–24-nt small RNAs mapped to the known miRNAs while the other size of small RNAs mostly mapped to rRNAs and tRNAs. This result implies that miRNAs are precisely processed to specific sizes by DCL enzymes while most of small RNAs originated from rRNA and tRNAs are degraded byproducts.

Table 1 Mapping of small RNA-seq reads to the *Arabidopsis thaliana* genome

Read length category	21–24 nt		Not 21 ~ 24 nt	
Feature category	% Aligned to the sense strand of the feature[a]	% Aligned to the antisense strand of the feature[a]	% Aligned to the sense strand of the feature[a]	% Aligned to the antisense strand of the feature[a]
Non-coding RNA gene				
miRNA	13.02 ± 1.21	0.01 ± 0.00	3.78 ± 0.53	0.00 ± 0.00
other_RNA	0.71 ± 0.08	0.63 ± 0.04	0.21 ± 0.02	0.05 ± 0.00
rRNA	4.85 ± 0.43	0.07 ± 0.01	7.84 ± 0.55	0.02 ± 0.00
snoRNA	0.05 ± 0.01	0.00 ± 0.00	0.24 ± 0.05	0.00 ± 0.00
snRNA	0.01 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.00 ± 0.00
tRNA	0.31 ± 0.02	0.02 ± 0.00	4.73 ± 1.11	0.03 ± 0.01
Protein-coding gene	2.98 ± 0.10	2.40 ± 0.11	1.26 ± 0.02	0.65 ± 0.09
Psuedogene	0.16 ± 0.00	0.14 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
Transposable element (TE) gene	8.57 ± 0.54	9.09 ± 0.59	0.86 ± 0.09	1.61 ± 0.18
Intergenic	15.52 ± 0.44		14.85 ± 0.33	
Reads aligned to genome	58.54 ± 0.84		36.19 ± 0.87	
Reads in each length category	61.51 ± 0.86		38.49 ± 0.86	
Total reads	100 (178,952,778 reads)			

[a]Mean ± standard deviation of 6 samples (3 control and 3 BR-treated samples)

We also noticed that a significant amount of small RNAs were mapped to protein-coding genes, which might play a role in gene expression regulation. In addition, we found that transposable elements and intergenic regions also accumulate small interfering RNAs (siRNAs) to suppress their expression.

Identification and differential expression analysis of BR-responsive small RNA clusters

Small RNA expression is regulated by various environmental changes and during the development (Baulcombe and Dean 2014; Bologna and Voinnet 2014; Khraiwesh et al. 2012). To measure the small RNA expression levels of epiBL-treated and control seedlings in Arabidopsis, a sliding bin of 100-bp divided the Arabidopsis genome and the abundance of 21–24-nt small RNAs that mapped in each bin were counted. Of 1.35 million bins of Arabidopsis genome, 79,781 bins (5.88%) represented more than 10TP4M expression levels in at least one small RNA library and used for further analysis. The most abundant small RNA clusters were identified in the loci encoding miR165/miR166 family and followed by other miRNA loci. To identify BR-responsive small RNA clusters, small RNA abundance in each bin was compared between epiBL-treated and control seedlings. Overall, a linear regression between two samples produced a coefficient of determination (R^2) of 0.9893, illustrating tight regulation of small RNA expression (Fig. 1a, b). With the statistical analysis, we have identified 38 bins with significant fold changes by BL treatment (Fig. 1a, b, Table 2). These include 30 down-regulated bins and eight up-regulated bins. Down-regulated bins correspond to miRNA loci, transposable elements, protein-coding genes, pseudogenes and others (Table 2). For instance, *At1g31173* encoding MIR167d represents down-regulation of mature miRNAs by epiBL treatment (Fig. 1c). We noticed that *At4g04408* and *At4g04409* are annotated as pseudogenes that are inverted repeats encoding MIR841b, which is down-regulated by epiBL-treatment (Fig. 1d). Of up-regulated bins, one is MIR156g locus and seven bins correspond to a *BRI1* gene. Especially, a lot of small RNAs were accumulated in a *BRI1* gene from both control and epiBL-treated seedlings. In a recently published database of 2000 Arabidopsis small RNA libraries, we were not able to see a significant accumulation of small RNAs in a *BRI1*gene (Feng et al. 2020). However, our small RNA sequencing data are from the transgenic plants expressing a BRI1-Flag protein under its native promoter in the *bri1-5* mutant. Thus, we assumed that these might be due to transgene-induced small RNA accumulation.

Correlation of BR-responsive small RNA clusters and mRNA expressions

To examine the correlation of small RNA abundance and mRNA expression levels in differentially expressed small RNA clusters, we have analyzed the expression patterns of three selected genes. mRNA expression patterns by epiBL-treatment in Arabidopsis seedlings were obtained from the previously published RNA-seq data (Oh et al. 2014). *At1g31173*, which encodes MIR167d, accumulated

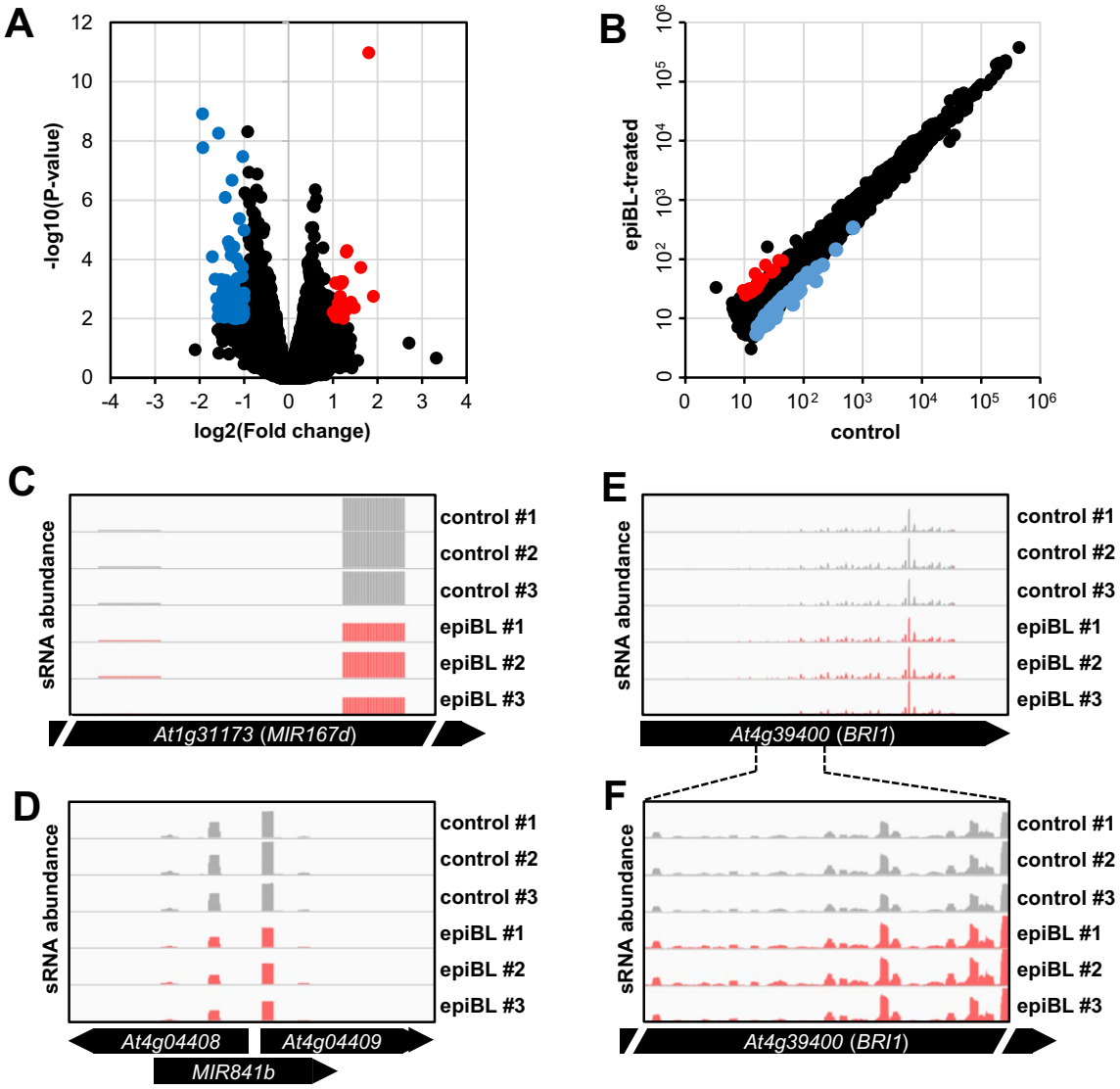

Fig. 1 Brassinosteroid-responsive siRNA clusters. **a** Volcano plot showing statistical significance ($-\log_{10}$ P value) versus fold change ($\log_2$ fold change) of small RNA cluster data from control and epiBL-treated seedlings (n = 3 biological replicates). Small RNA clusters with increased expression (fold-change value > 2 and p value < 0.01) are shown in red, and small RNA clusters with decreased expression (fold-change value < 2 and p value < 0.01) are shown in blue. **b** Scatter plot illustrating pairwise comparison of the normalized abundance of small RNA clusters between control and epiBL-treated seedlings. Each dot represents the mean of the normalized abundance of a small RNA cluster (n = 3 biological replicates). Red and blue dots indicate up-regulated and downregulated sRNA clusters, respectively. **c–f** Snapshots of the IGV browser showing the position and abundance of small RNAs for the up-regulated and down-regulated siRNA clusters. Small RNA abundances for three biological replicates of control and BL-treated samples are indicated with grey and magenta colors, respectively (color figure online)

less small RNAs under epiBL treated condition (Fig. 1c). Although *MIR167d* expression was also slightly down-regulated by epiBL treatment, the regulation was not significant because the expression levels were quite low (less than 2 FPKM) both in control and epiBL-treated seedlings (Fig. 2a). *At3g58270* encoding Phospholipase-like protein also did not represent significant expression changes by epiBL treatment even though small RNAs originated in this gene were significantly down-regulated (Fig. 2b). However, *At4g39400*, which encodes BRI1, represented a significant negative correlation between small RNA expression and mRNA expression by epiBL treatment (Fig. 2c). Small RNAs originated from a *BRI1* transgene were more accumulated while *BRI1* mRNAs were down-regulated by epiBL treatment. It is known that the expression of *BRI1*, encoding a BR receptor, is down-regulated by a BR-mediated negative feedback mechanism. This result implies that up-regulation of small RNAs in a

Table 2 Differentially expressed small RNA clusters by epiBL treatment

Bin ID	Chr	Bin start	Bin end	Feature	Feature category	Feature annotation	Mean control	Mean BR	$\log_2$ (BR/Control)	FDR
pBin0102271	Chr1	10,227,001	10,227,100	AT1G29265	miRNA	MIR399A	346	205	− 0.75	1.E− 02
pBin0111379	Chr1	11,137,801	11,137,900	AT1G31173	miRNA	MIR167D	162	43	− 1.93	3.E− 04
pBin0122948	Chr1	12,294,701	12,294,800	AT1G33860	protein_coding	unknown protein	148	75	− 0.99	4.E− 03
pBin0314687	Chr2	1,040,901	1,041,000	AT2G03445	miRNA	MIR398A	349	145	− 1.27	2.E− 03
mBin0388404	Chr2	8,412,601	8,412,700	AT2G19425	miRNA	MIR156G	23	79	1.80	8.E− 07
pBin0582342	Chr3	8,108,101	8,108,200	AT3G22886	miRNA	MIR167A	57,460	34,400	− 0.74	3.E− 02
pBin0643070	Chr3	14,180,901	14,181,000	AT3TE58155	TE	ATDNA12T3_2	79	29	− 1.42	4.E− 03
mBin0658027	Chr3	15,676,601	15,676,700	AT3TE63395	TE	DNA/En-Spm	984	580	− 0.76	1.E− 02
mBin0658028	Chr3	15,676,701	15,676,800	AT3TE63395	TE	DNA/En-Spm	135	72	− 0.89	5.E− 02
pBin0658034	Chr3	15,677,301	15,677,400	AT3TE63395	TE	DNA/En-Spm	911	559	− 0.71	1.E− 03
mBin0658037	Chr3	15,677,601	15,677,700	AT3TE63405	TE	DNA/En-Spm	359	208	− 0.79	4.E− 03
mBin0658038	Chr3	15,677,701	15,677,800	AT3TE63405	TE	DNA/En-Spm	117	58	− 1.01	3.E− 02
pBin0658039	Chr3	15,677,801	15,677,900	AT3TE63405	TE	DNA/En-Spm	230	134	− 0.78	2.E− 02
pBin0658040	Chr3	15,677,901	15,678,000	AT3TE63405	TE	DNA/En-Spm	221	116	− 0.93	4.E− 03
mBin0717035	Chr3	21,577,401	21,577,500	AT3G58270	protein_coding	Phospholipase-like protein	66	17	− 1.94	5.E− 05
pBin0757702	Chr4	2,184,201	2,184,300	AT4G04408	pseudogene	pseudogene	685	336	− 1.03	4.E− 04
pBin0757703	Chr4	2,184,301	2,184,400	AT4G04408	pseudogene	pseudogene	3034	1729	− 0.81	9.E− 03
pBin0757704	Chr4	2,184,401	2,184,500	AT4G04409	pseudogene	Pseudogene of AT3G54560	5288	3602	− 0.55	2.E− 02
pBin0757721	Chr4	2,186,101	2,186,200	AT4G04409	pseudogene	Pseudogene of AT3G54560	109	51	− 1.11	1.E− 02
mBin0788542	Chr4	5,268,201	5,268,300	AT4G08345	tRNA	tRNA-Leu (anticodon: TAG)	426	229	− 0.89	1.E− 03
pBin0814317	Chr4	7,845,701	7,845,800	AT4G13493	miRNA	MIR850a	1325	701	− 0.92	1.E− 04
pBin0918913	Chr4	18,305,301	18,305,400	AT4G39363	snoRNA	snoRNA	386	229	− 0.75	5.E− 02
pBin0919120	Chr4	18,326,001	18,326,100	AT4G39400	protein_coding	BRI1	615	950	0.63	4.E− 03
mBin0919120	Chr4	18,326,001	18,326,100	AT4G39400	protein_coding	BRI1	683	994	0.54	2.E− 02
mBin0919121	Chr4	18,326,101	18,326,200	AT4G39400	protein_coding	BRI1	1229	1855	0.59	3.E− 03
mBin0919125	Chr4	18,326,501	18,326,600	AT4G39400	protein_coding	BRI1	5609	8193	0.55	6.E− 03
pBin0919125	Chr4	18,326,501	18,326,600	AT4G39400	protein_coding	BRI1	1880	2798	0.57	4.E− 02
mBin0919126	Chr4	18,326,601	18,326,700	AT4G39400	protein_coding	BRI1	1198	1785	0.58	7.E− 03
pBin0919127	Chr4	18,326,701	18,326,800	AT4G39400	protein_coding	BRI1	4703	6681	0.51	2.E− 02
mBin0956277	Chr5	3,456,601	3,456,700	AT5G10945	miRNA	MIR156D	353	208	− 0.76	1.E− 02
mBin1191955	ChrC	48,801	48,900	ATCG00420	protein_coding	NADH dehydrogenase subunit J	78	26	− 1.58	1.E− 04
pBin1192211	ChrC	74,401	74,500	ATCG00710	protein_coding	photosystem II reaction center H	773	470	− 0.72	3.E− 03
pBin1192476	ChrC	100,901	101,000	ATCG00920	rRNA	chloroplast-encoded 16S ribosomal RNA	904	504	− 0.84	5.E− 02
pBin1192519	ChrC	105,201	105,300	ATCG00950	rRNA	chloroplast-encoded 23S ribosomal RNA	884	552	− 0.68	2.E− 02
pBin1192609	ChrC	114,201	114,300	ATCG01030	tRNA	tRNA-Leu	399	217	− 0.88	5.E− 03
mBin1192796	ChrC	132,901	133,000	ATCG01180	rRNA	chloroplast-encoded 23S ribosomal RNA	599	384	− 0.64	3.E− 02

Table 2 (continued)

Bin ID	Chr	Bin start	Bin end	Feature	Feature category	Feature annotation		Mean control	Mean BR	$\log_2$ (BR/Control)	FDR
mBin1192807	ChrC	134,001	134,100	ATCG01190	tRNA	tRNA-Ala		5370	3485	− 0.62	4.E− 03
pBin1193481	ChrM	46,901	47,000	N/A	n/a	n/a		1267	851	− 0.57	3.E− 02

down-regulated transcript might play a role in epigenetic regulation of the transgene.

Identification of differential expression analysis of BR-responsive miRNAs

To identify BR-responsive miRNAs, we have examined the expression levels of mature miRNAs in the control and epiBL-treated seedlings. Of 428 Arabidopsis mature miRNAs, registered in miRBase Release 22.1, 257 miRNAs were detected in our small RNA libraries. Of 257 expressed miRNAs, only two mature miRNAs, miR156g and miR398a-5p, were significantly regulated by epiBL treatment (Fig. 3a, b). This result implies that most miRNAs may not be regulated by BR. It is also possible that treatment of epiBL for 90 min may not be enough to change the other miRNA expressions. We also noticed that some miRNAs that we have identified from small RNA cluster analysis were not classified as BR-responsive miRNAs. This might be due to using the different criteria for identifying the differentially expressed small RNAs between small RNA cluster analysis and mature miRNA analysis. It is also possible that mature miRNAs were not significantly changed even though the other small RNAs in a precursor, including miRNA* (the passenger strand of mature miRNA) and other degraded small RNAs, were slightly changed by epiBL-treatment. Thus, we did not count miR167d, miR399a, miR841ab, and miR850 as BR-responsive miRNAs (Table 3). In the end, miR156g and miR398a-5p were identified as up-regulated and down-regulated miRNAs by epiBL treatment (Fig. 3c, d).

The effect of BR-responsive miRNAs on their target gene expression

To assess the effects of differentially expressed miRNAs on their target gene expression, BR responses of miRNA target gene expressions were examined by analyzing the RNA-seq data. miR156g targets seven genes encoding Squamosa promoter binding protein-like (SPL) proteins, including SPL2, SPL3, SPL4, SPL6, SPL9, SPL11, and SPL15 (Jeong et al. 2013b; Wu and Poethig 2006). Because miR156g are up-regulated by epiBL treatment, we expected that *SPL* genes could be down-regulated by BR. Of seven *SPL* targets, however, only *SPL3* showed significant down-regulation by epiBL treatment (Fig. 4a). This result might be due to the fact that there are ten other miR156 family members which are not regulated by BRs (Table 3). Of miR156 family, miR156a-f and miR157a-c are more abundant than miR156g. The contribution of up-regulated miR156g by BRs may not be enough to affect the expression levels of the other *SPL* genes except *SPL3*. However, down-regulation of SPL3 by BRs might be miR156g-specific. It is known that different members

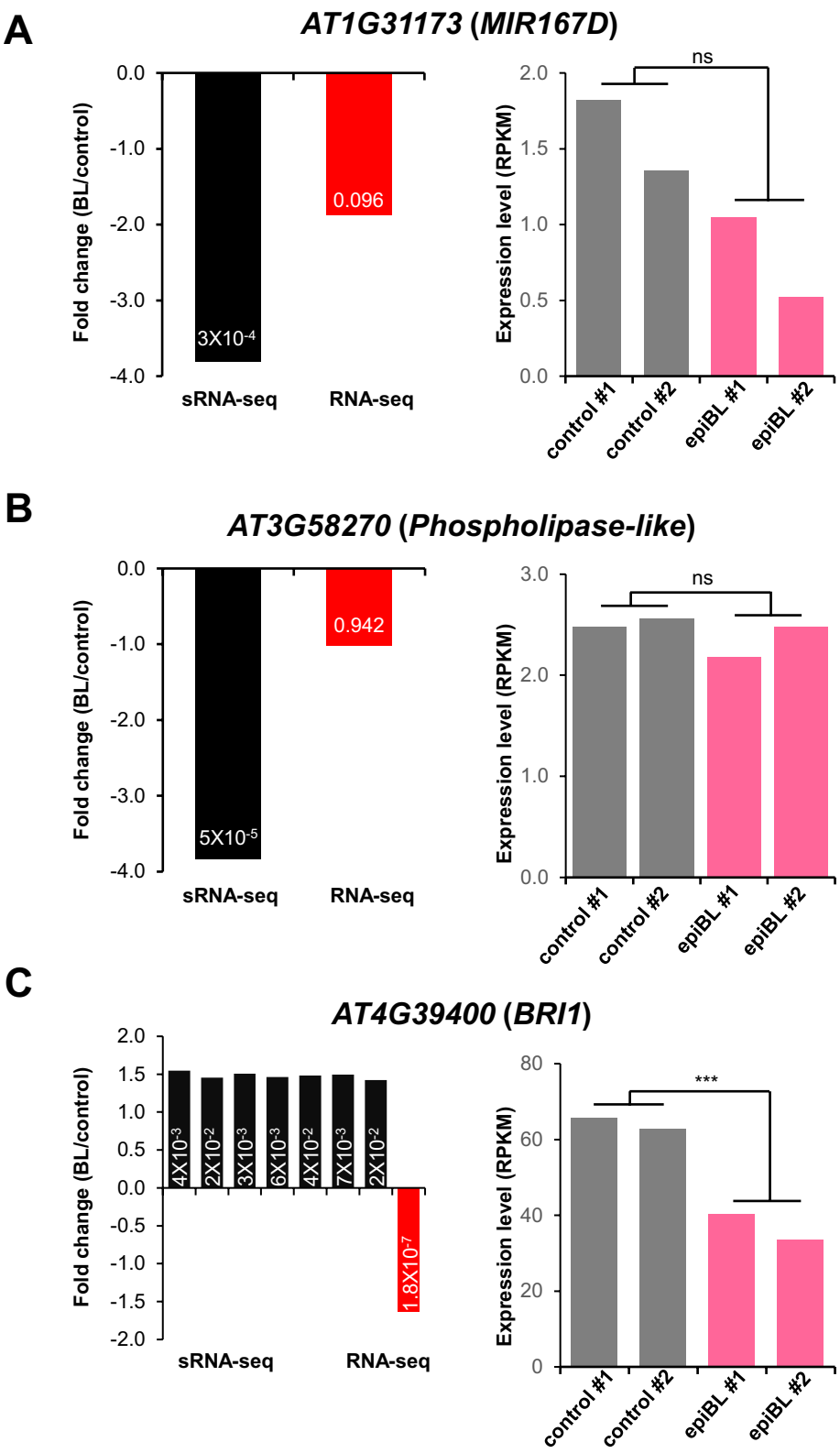

Fig. 2 Small RNA abundances and mRNA expression levels of brassinosteroid-responsive siRNA clusters. **a** Small RNA and mRNA expression patterns of *At1g31173* encoding MIR167D. **b** Small RNA and mRNA expression patterns of *At3g58270* encoding Phospholipase-like protein. **c** Small RNA and mRNA expression patterns of *At4g39400* encoding BRI1. On the left panel, fold changes of small RNA abundance and mRNA expression levels between control and epiBL-treated seedlings are shown as black and red bars, respectively. P values of the statistical significance are indicated in the bars. On the right panel, the mRNA expression levels of sRNA clusters are shown. Two biological replicates of control and epiBL-treated seedlings are represented as grey and magenta colors, respectively. RPKM; Reads Per Kilobase Million (color figure online)

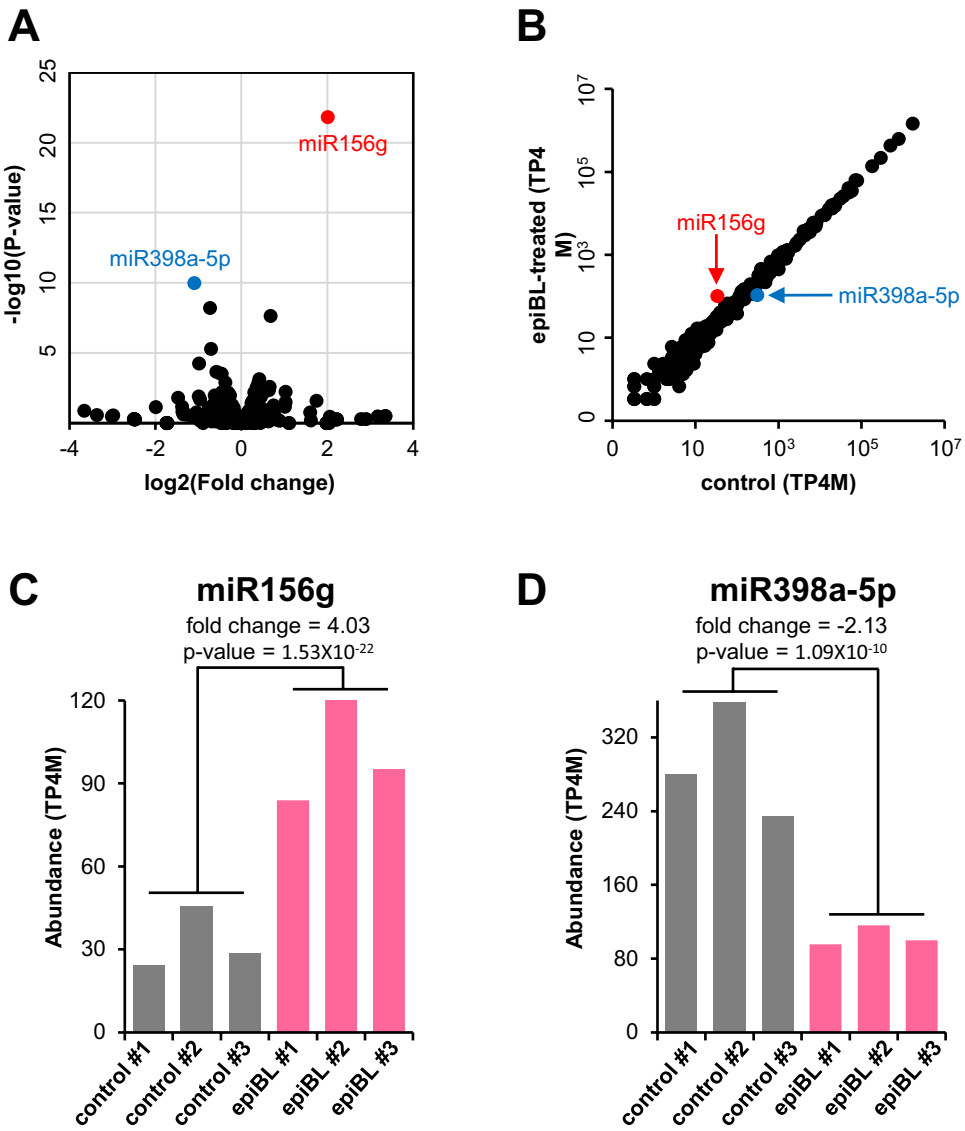

Fig. 3 Brassinosteroid-responsive miRNAs. **a** Volcano plot indicating statistical significance ($-\log_{10}$ P value) versus fold change ($\log_2$ fold change) of miRNA expression levels from control and epiBL-treated seedlings (n = 3 biological replicates). A miRNA with increased expression (fold-change value > 2 and p value < 0.01) is shown in red, and a miRNA with decreased expression (fold-change value < 2 and p value < 0.01) is shown in blue. **b** Scatter plot showing pairwise comparison of the normalized abundance of miRNA between control and epiBL-treated seedlings. Each dot represents the mean of the normalized abundance of a miRNA (n = 3 biological replicates). Red and blue dots indicate up-regulated and down-regulated miRNAs, respectively. **c**, **d** Expression levels of miR156g and miR398a-5p between control and BL-treated seedlings. Expression data are from three biological replicates of small RNA-seq with the normalized miRNA expression levels (TP4M, Transcripts Per 4 Millions). Fold changes and p values are indicated for the significance of deferential expression (color figure online)

of a miRNA family could be differentially expressed and may have their distinct function on their targets (Jeong 2016; Neilsen et al. 2012). Further study on the expression patterns of miR156 family members and *SPL* genes may provide the insight into the mechanism of BR-specific miR156g/SPL3 regulation. Since the miR156/SLP3 module is known to regulate vegetative phase change and flowering (Gandikota et al. 2007; Wu and Poethig 2006), BRs may regulate shoot development in Arabidopsis by miR156 and *SPL* genes.

We have identified miR398a-5p as a down-regulated miRNA. In Arabidopsis, there are three MIR398 precursors, MIR398a, MIR398b, and MIR398c. Of these, MIR398b and MIR398c generates same mature miR398bc and less abundant miR398bc-5p. However, miR398a generates less abundant miR398a and more abundant miR398a-5p. miR398a sequence is different in the 3′ end compared to miR398bc sequence (Table 3). miR398 is known to target two *copper/zinc superoxide dismutase* genes, *CSD1* and *CSD2* (Beauclair et al. 2010; Jones-Rhoades and Bartel 2004). Because the expression of miR398a and miR398bc were not affected by BRs, the expression levels of *CSD1* and *CSD2* were affected by epiBL treatment (Fig. 4b). The expression levels of the predicted targets of miR398a-5p, genes encoding ARM repeat and ABCG40, were also not significantly changed by epiBL treatment (Fig. 4b). This result implies that these two targets are not regulated by miR398a-5p or that miR398a-5p may not be a functional

Table 3 Expression patterns of differentially expressed miRNAs and their family members

miRNA	Sequence	Control (TP4M)	BL (TP4M)	Fold change	p value
miR156a-f	–UGACAGAAGAGAGUGAGCAC	15,252.3	12,338.9	1.03	0.709
miR156g	–**C**GACAGAAGAGAGUGAGCAC	31.6	100.1	4.03	$1.53*10^{-22}$
miR156h	-UGACAGAAGA**A**AGA**G**AGCAC	5.6	5.9	1.33	0.633
miR157a -c	**U**UGACAGAAGA**U**AGA**G**AGCAC	28,551.9	22,263.1	− 1.01	0.798
miR157d	–UGACAGAAGA**U**AGA**G**AGCAC	1604.9	1124.1	− 1.13	0.117
miR398a	UGUGUUCUCAGGUCACCCCUU	75.3	61.5	1.04	0.728
miR398bc	UGUGUUCUCAGGUCACCCCU**G**	270,334.0	213,708.3	− 1.01	0.927
miR398a-5p	AAGGAGUGGCAUGUGAACACA	286.4	104.1	− 2.13	$1.09*10^{-10}$
miR398bc-5p	AGGGUUGAUAUGAGAACACAC	670.4	487.7	− 1.10	0.218
miR167ab	UGAAGCUGCCAGCAUGAUCUA	4820.3	4088.8	1.02	0.822
miR167c	–**U**AAGCUGCCAGCAUGAUCUUG	173.9	130.8	− 1.05	0.707
miR167d	UGAAGCUGCCAGCAUGAUCU**G**G	5334.5	3485.2	− 1.23	0.011
miR399a	UGCCAAAGGAGAUUUGCCC**U**G	7311.1	4808.2	− 1.20	0.011
miR399bc	UGCCAAAGGAGA**G**UUGCCC**U**G	17,672.1	15,123.9	1.05	0.497
miR399d	UGCCAAAGGAGAUUUGCCCCG	582.7	357.8	− 1.30	0.001
miR399e	UGCCAAAGGAGAUUUGCC**U**CG	9.6	5.3	− 1.44	0.357
miR399f	UGCCAAAGGAGAUUUGCCC**G**G	958.7	622.5	− 1.23	0.007
miR841a	UACGAGCCACUUGAAACUGAA	4068.4	3339.8	1.07	0.450
miR841b	UACGAGCCACU**G**GAAACUGAA	936.7	442.8	− 1.63	$5.25*10^{-6}$
MIR850a	UAAGAUCCGGACUACAACAAAG	104.8	71.7	− 1.16	0.253

miRNA. We can not rule out the possibility that miR398a-5p may regulate the other targets that were not computationally predicted.

Conclusion

In this study, we have examined the BR-responsive small RNA clusters and miRNAs by analyzing small RNA libraries constructed from control and epiBL treated seedlings. In results, 38 differentially expressed small RNA clusters and two BR-responsive miRNAs were identified. Of BR-responsive small RNA clusters, we found that a *BRI1* transgene generates various small RNAs, which were not identified in the wild type plants. In addition, these small RNAs were up-regulated by epiBL treatment while *BRI1* expression is down-regulated by a negative feedback mechanism. This result implied the possible epigenetic regulation in a transgene by BRs. Of BR-responsive miRNAs, we were not able to see the effect of BR-repressed miR398b on their target gene expression. However, BR-induced miR156g down-regulated *SPL3*. These data will provide the insight into how small RNAs are involved in BR responses in plants.

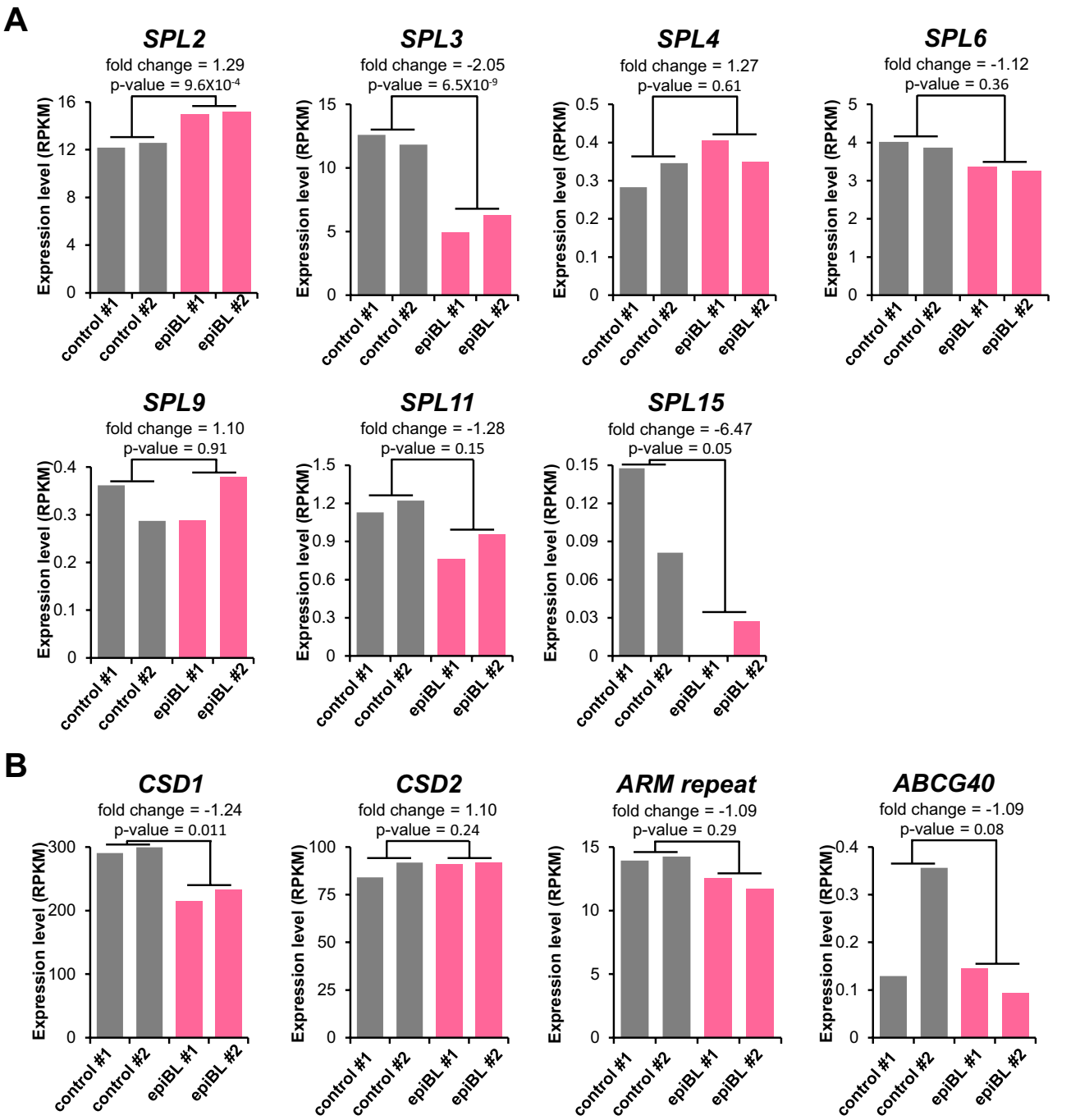

Fig. 4 Analysis of miRNA target gene expression between control and BL-treated seedlings. **a** Expression levels of miR156g target genes, *SPL2*, *SPL3*, *SPL4*, *SPL6*, *SPL9*, *SPL11*, and *SPL15*. **b** Expression levels of miR398a-3p and miR398a-5p targets. *CSD1* and *CSD2* are targets of miR398a-3p, while *ARM repeat* and *ABCG40* are predicted targets of miR398a-3p. miRNA target gene expression levels are examined by analyzing the RNA-seq data of control and epiBL-treated seedlings. Histograms show the normalized RPKM (Reads Per Kilobase of transcript, per Million mapped reads) values for two biological replicates of control and BL-treated seedlings. The fold changes and p values between control and epiBL-treated seedlings are indicated (color figure online)

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Data availability The small RNA sequencing dataset generated in this study has been deposited at Gene Expression Omnibus (GEO) of National Center for Biotechnology Information (NCBI) under the accession number GSE149360.

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