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High-throughput sequencing and differential expression analysis of miRNAs in response to Brassinosteroid treatment in *Arabidopsis thaliana*

Gunjan Sirohi[1] · Ankitha Khandelwal[1] · Meenu Kapoor[1]

Abstract

Brassinosteroids are a class of phytohormones that play crucial roles in improving stress tolerance in plants. Many biochemical and physiological changes in response to abiotic stress are related to regulation of gene expression and accumulation of associated proteins. MicroRNAs (miRNAs) are class of small non-coding RNAs that regulate gene expression post-transcriptionally. Roles of these regulatory RNAs in brassinosteroid (BR) signalling have however remained elusive. In this study using high-throughput small RNA sequencing method, we present a comprehensive compilation of BR-induced differentially expressed microRNAs in root and shoots of *Arabidopsis thaliana* seedlings. We identified 229 known miRNAs belonging to 102 families and 27 novel miRNAs that express in response to exogenous BR treatment. Out of 102 families, miRNAs belonging to known 48 families and out of 27 novel miRNAs, 23 were observed to be differentially expressed in response to BR treatment. Among the conserved miRNAs, all members of miR169 were observed to be downregulated in both shoot and root samples. While, auxin-responsive factors were predicted to be direct targets of some novel miRNAs that are upregulated in shoots and suppressed in roots. The BR-responsive tissue-specific miRNome characterized in this study can be used as a starting point by investigators for functional validation studies that will shed light on the underlying molecular mechanism of BR-mediated stress tolerance at the level of post-transcriptional gene regulation.

Keywords Brassinosteroid · MicroRNAs · Small RNA sequencing · *Arabidopsis thaliana* · GenBank accession no. SRP152755

Introduction

Brassinosteroids (BRs) are a class of polyhyroxylated steroidal hormones that function as growth-promoting molecules and occur widely across the plant kingdom. A number of studies have shown that BRs promote cell division and expansion; regulate senescence, male sterility, pollen development and fruit ripening; and modulate plant responses under numerous biotic and abiotic stresses (Sreeramulu et al. 2013; Sharma

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et al. 2015; Alptekin et al. 2017; Anwar et al. 2018). Studies on interactive roles of BR-responsive elements with transcription factors and signalling components of other phytohormones are also documented (Choudhary et al. 2012; Gruszka 2013; Divi et al. 2015; Yuan et al. 2015). Recently, it has been shown that drought/dehydration stress induces miRNA expression in a tissue-specific manner, and comprehensive data on understanding miRNomes in members of *Triticeae* and other grasses has been compiled (Budak and Akpinar 2011; Akpinar et al. 2015; Alptekin et al. 2017). Exogenous application of BR is known to increase tolerance in plants towards various abiotic stresses (temperature, water, salt, radiation and heavy metals) by improving their photosynthetic capacity, increasing activities of antioxidant enzymes and affecting other physiological processes (Anuradha and Rao 2001; Yuan et al. 2012; Wei et al. 2015; Li et al. 2016). Majority of these biochemical and physiological changes are a reflection of underlying alterations in transcript abundance and their associated proteins. Hence, an understanding of post-transcriptional gene regulatory processes is crucial for

gaining insight into the underlying molecular mechanisms of stress tolerance in plants. In this study, we have focused on establishing a direct link between BR and miRNA induction that will provide insight into microRNA-BR crosstalk in *A. thaliana* seedlings.

MicroRNA-mediated post-transcriptional regulation of gene expression is crucial for both stress recognition and responses (Mallory and Vaucheret 2006; Ergen and Budak 2009; Budak et al. 2015). A number of studies in *Arabidopsis* and other plants have demonstrated the importance of miRNA-based stress responses where defects in miRNA metabolism and their biogenesis cause plants to become hypersensitive to abiotic stress-associated hormones such as abscisic acid (Lu and Fedoroff 2000; Kusumanjali et al. 2012; Kumari et al. 2013; Gim et al. 2014; Sirohi et al. 2018). To date, a number of in silico and experimental studies on drought, UV and heat stresses have identified and characterized abiotic stress-associated miRNAs (Budak and Akpinar 2011). However, direct involvement of phytohormones such as BR in modulating miRNA expression at genome-wide levels has not been studied. Though, recently regulation of root development in *Populus euphratica* by ABA-responsive miRNA processes was reported. This study provided insight into crosstalk between ABA regulation of root growth through miRNA-mediated pathways (Lian et al. 2018). Since mechanism of BR regulation especially through miRNA-mediated pathways is poorly understood so, in this study, we report tissue-specific BR-responsive miRNA population using the next-generation sequencing method.

Small RNA sequencing is a powerful tool for genome-wide identification and quantification of small RNA species in plants. This methodology has opened up tremendous possibilities to explore involvement of small RNAs in response to various treatments in plant species as it helps in identifying large number of small RNAs in short time duration (Song et al. 2010; Ge et al. 2013). Using this powerful technique, large body of data has been generated in numerous plant species (Xu et al. 2010; Wang et al. 2011; Xie et al. 2017).

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana, ecotype Columbia (Col-0), was used as the plant material. For analysing BR dose-dependent effects on seed germination and seedling growth, four different concentrations of 24-epibrassinolide (Sigma) 0 nM, 10 nM, 100 nM and 1 μM were selected on the basis of literature study. Stock solution (10^{-2} M) of 24-epibrassinolide (Sigma) was prepared in 50% (*v/v*) ethanol. Seeds were surface sterilized with 1% bleach (sodium hypochlorite) and 0.5% Triton X-100 followed by five to six times washing with sterile

water. These were then incubated at 4 °C for 48 h for vernalization. Imbibed seeds were inoculated (20 seeds/plate) on one-half-strength Murshige and Skoog (MS) plates prepared with 1% sucrose and 0.8% agar and supplemented with different concentrations of BR (0 nM, 10 nM, 100 nM and 1 μM). Plates were oriented vertically and placed in climate-controlled conditions (22 °C ± 2 °C temperature, 55% ± 5% relative humidity with 16-h light of intensity 60–90 μmol/ m^2 s and 8-h dark cycle). Throughout the experiment, petri plates were sealed with gas-permeable tapes to avoid ethylene accumulation. Seed germination was visually scored after 2 days, and percent germination was calculated. Images were captured using stereozoom microscope (Olympus, SZX16). For analysing effects on seedling growth, 5-day-old seedlings propagated on one-half-strength MS plates were transferred to fresh plates supplemented with 0 nM, 10 nM, 100 nM and 1 μM BR. To monitor the effects on primary root elongation, root tips were marked with a black dot immediately after transfer to BR-supplemented plates. Morphological analysis was performed after 7 days of growth on these plates. For biomass calculations, fresh weight of 30 seedlings per treatment was determined. Images of seedlings were captured using Canon D70 DSLR camera. Reproducibility and authenticity of dose-dependent experiments were confirmed by repeating the experiments thrice.

Superoxide dismutase assay

Total protein was extracted from 100 mg tissue using extraction buffer [sodium phosphate buffer (100 mM, pH 7.0), EDTA (0.4 mM), glycerol (10%), $Mgcl_2$ (5 mM), phenazinemetho sulfate (1 mM), dithiothreitol (2 mM) and HEPES (50 mM, pH 7.5)]. The homogenate was transferred to fresh tubes and centrifuged at 10,000 rpm for 20 min at 4 °C. Protein concentration in supernatants obtained from each sample was estimated using Bradford method. Superoxide dismutase (SOD) activity was analysed following protocol by Kakkar et al. (1984). Briefly, reaction mixture containing 1.2 ml of 0.052 M tetrasodium pyrophosphate buffer (pH 8.3), 0.3 ml of 1 mM phenazinemetho sulfate (PMS) and 0.2 ml of 1 mM nitroblue tetrazolium (NBT), 0.2 ml of 1 mM NADH and 1 ml distilled water were mixed. To this, 0.1 ml of protein extract was added and the reaction was allowed to occur at 30 °C for 90 s. Reaction was stopped by adding 1 ml glacial acetic acid. Mixture was vigorously shaken after adding 4 ml of n-butanol and after 10-min incubation was centrifuged at 10,000 rpm for 5 min. The n-butanol layer was taken, and colour intensity of the chromogen in this fraction was measured at 560 nm against n-butanol taken as control (blank). SOD activity was measured and expressed as unit per milligram of protein.

Small RNA library construction, sequencing and data mining

For small RNA analysis, seedlings grown for 7 days on 100-nM BR-supplemented plates were harvested. Roots and shoots were separately sampled and collected as No BR-R and BR-R (for roots) and No BR-S and BR-S (for shoots) and processed for small RNA sequencing. Total RNA was isolated using TRiZol reagent (Invitrogen, USA). Small RNA library was prepared using the Small RNA Sample Preparation Kit (Illumina Technologies) following manufacturer's instructions. Libraries were sequenced on Illumina HiSeq 4000 platform. Raw reads generated were first subjected to NGS QC Toolkit to remove low-quality reads keeping 70% length cut-off and a Phred score of 20 without adapter filtering. Adapter sequences were then removed from the filtered reads using Cutadapt tool (Illumina Small RNA 3′ Adapter). The adapter-trimmed reads were again subjected to NGS QC Toolkit to obtain a final set of adapter-trimmed high-quality (HQ) reads keeping 80% length cut-off and a Phred score of 30. The data so obtained contained > 99% high-quality reads with good phred score and was further analysed. Unique tags (non-redundant reads), abundance and read length of the HQ reads were then calculated using AWK Script. The sequenced dataset generated in this study has been deposited at Sequence Read Archive (SRA) of National Center for Biotechnology Information (NCBI) under the accession number SRP152755.

Data analysis

Pre-processed and cleaned sequence data was further analysed. Unique tags in the HQ reads were subjected to BLASTN against a curated RNA central database (RNA central database without miRNA, pre-miRNA and miRNA-like molecules). The unmapped tags were annotated using Rfam ID and Rfam class and categorized as different types of small non-coding RNAs like rRNA, tRNA, snoRNA, snRNA, etc. These non-coding RNAs and other classes of RNAs were removed from the sRNA sequences. Remaining unmapped reads were subjected to size filtering using 16–28-nt filter. These size-filtered reads were the putative miRNA candidates and were taken forward for miRNA identification. These reads were subjected to UEA Small RNA Workbench. miRProf analysis was undertaken for identification of known miRNAs. For this, alignments were performed against the miRBase database (Release 21; http://www.mirbase.org; Kozomara and Griffiths-Jones 2011). Conserved/known miRNAs were identified from perfectly matched sequences. For identification of novel miRNA, miRCat analysis was done using *Arabidopsis thaliana* genome as reference. Conditions for filtering miRCat result and identification of novel miRNAs included (a) number of consecutive mismatches between

miRNA and miRNA* not more than 3, (b) loci must contain no more than four non-overlapping sRNAs and (c) hairpin must be at least 50 nt in length. Candidate pre-miRNAs were identified by folding the flanking genome sequences of distinct miRNAs using MIREAP followed by prediction of secondary structures using mFold v3.5 (Zuker 2003). Criteria used for stem-loop hairpin and genome mapping were similar to that mentioned in Meyers et al. (2008).

Intersample and intrasample normalization was performed, and absolute expression levels of miRNAs in each library were calculated. For differential expression analysis, absolute expression levels of miRNAs were compared between two libraries. Differences in expression were calculated using $\log_2$ fold change of absolute expression levels in different samples. MicroRNAs showing ≥ 1 fold change were considered as differentially expressed and were carried for further analysis.

Target prediction and functional classification based on GO analysis

Once a final set of miRNAs were obtained, potential targets of differentially expressed miRNAs were identified using psRNATarget server (http://plantgrn.noble.org/psRNATarget; Dai and Zhao 2011). Gene ontology (GO) analysis was performed to classify gene features with respect to molecular functions, cellular components, biological processes and biological pathways.

Validation of BR-responsive miRNAs and target gene expression

For validating expression of BR-responsive miRNAs identified as differentially expressing in NGS study, stem-loop RT-qPCR method was used, while for validating expression of target genes, RT-qPCR method was employed. Total RNA isolated from tissue samples sent for small RNA sequencing were used as templates for expression studies. Experiments were performed using three biological replicates with three technical replicates for each biological sample. Stem-loop RT-qPCR was performed using SYBR Green (Fermentas, USA) as per protocol mentioned in Gasic et al. (2007). Primers were designed according to previously described protocol (Chen et al. 2005). Quantitative variation between different samples was calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Amplification of 5SrRNA was used as internal control to normalize data. Primer sequences used for this study are listed in Supplementary Table 1.

For RT-qPCR of selected target genes, first-strand complementary DNA (cDNA) was synthesized using 1 μg of total RNA in a 20-μL reaction using the RevertAid First-Strand cDNA synthesis kit (Thermo Scientific) following manufacturer's protocol. Primers were designed preferentially from the

3′ ends of genes using NetPrimer software (http://www.premierbiosoft.com/netprimer) with default parameters (Supplementary Table 1). Forward and reverse primers at 5 μM were mixed with SYBR Green PCR master mix as per manufacturer's instructions. Reaction was carried out using the real-time PCR system (Agilent). To normalize variance among the samples, *A. thaliana* actin gene was used as endogenous control. Transcript levels for candidate genes in different samples were determined using the delta delta cycle threshold method (Livak and Schmittgen 2001). Relative expression values were calculated after normalizing against the maximum expression value.

Results

Dose-dependent effects of 24-epibrassinolide

To determine the optimal concentration of BR for analysing tissue-specific BR-responsive miRNA population, a dose-dependent study to analyse effects of BR on seed germination, seedling growth and activity of the antioxidant enzyme SOD was undertaken. It was observed that exogenous application of 0 nM, 10 nM, 100 nM and 1 μM BR did not affect germination efficiency of *A. thaliana* seeds (Fig. 1a). However, effects on hypocotyl elongation on 2-day-old seedlings were more prominent (Fig. 1a). As compared to seedlings on 0 nM BR, increased hypocotyl elongation was observed in light-grown seedlings at 10 nM, 100 nM and 1 μM concentration (Fig. 1a). Similar effects on hypocotyl growth have been previously reported in Arabidopsis seedlings grown on 10 nM, 100 nM and 1 μM BR-supplemented MS plates (Gupta et al. 2015). To see morphological effects at later stages of seedling development, 5-day-old seedlings grown on one-half-strength Murashige and Skoog media were transferred to fresh media plates supplemented with different BR concentrations (0 nM, 10 nM, 100 nM and 1 μM). It was observed that seedlings grown on 0 nM developed primary roots with fewer lateral roots. In contrast, seedlings propagated on BR-supplemented media developed longer primary roots that showed prominent curling (Fig. 1b). Interestingly, seedlings propagated on 100 nM BR showed more proliferative root and shoot growth as compared to seedlings grown on 0 nM, 10 nM or 1 μM BR. Determination of fresh weight showed that seedlings propagated on 100 nM BR responded maximally by accumulating highest biomass as compared to seedlings grown in other concentrations. Further, analysis of SOD activity in seedlings also showed maximal activation at 100 nM concentration (Fig. 1c). Hence, on the basis of positive effects of 100 nM 24-epibrassinolide on shoot and root growth and increased activity of SOD, 100 nM BR was selected for tissue-specific BR-responsive miRNA study.

Annotation of sequenced small RNA reads

Root and shoot samples from seedlings grown for 7 days on 100 nM BR-supplemented plates were separately harvested, and total RNA was isolated (Fig. 2). To perform small RNA sequencing, four libraries were prepared and sequenced using Illumina HiSeq platform. These included No-BR-R (root samples grown on MS media without BR), No-BR-S (shoot samples without BR), BR-R (root tissues with 100 nM exogenously supplied BR) and BR-S (shoot samples with 100 nM exogenously supplied BR). A total of 24,816,172, 25,902,123, 25,210,454 and 25,691,643 raw reads were obtained from No-BR-R, No-BR-S, BR-R and BR-S libraries, respectively (Table 1). After removal of low-quality reads and adaptor sequences, reads shorter than 15 nt and those with poly-A sequences accounted for 0.12% in No-BR-R, 0.13% in No-BR-S, 0.11% in BR-R and 0.15% in BR-S samples, respectively. Thus, 24 to 25 million clean reads of high-quality (> 99%) were obtained (Table 1). These high-quality reads were then annotated to ascertain the abundance of different categories of small RNAs in the four samples (Fig. 3a). Out of tRNAs, rRNAs and snRNAs, percent distribution of miRNAs in each of the four libraries was observed to be 31% in BR-R, 21% in BR-S, 20% in No-BR-R and 33% in No-BR-S (Fig. 3a). The range of length distribution of small RNA sequences was observed to be between 15 and 51 nucleotides with 23 and 24 nt small RNA sequences dominating the small RNA population (Fig. 3b). Among these, 23 nucleotide small RNAs constituted 15% in all the four libraries, while abundance of 24 nt RNAs was observed to be > 35% in No-BR-S, ~ 25% in No-BR-R, ~ 32% in BR-S and ~ 31.5% in BR-R samples, respectively. Mean frequencies of sRNAs among the libraries were taken as the average number of common sequence frequency which was observed to be 302.68, while library-specific reads varied from 1.37 in BR-R to 19.50 in BR-S (Table 2). While, frequencies of sRNAs in No-BR-R and No-BR-S were observed to be 1.45 and 1.53, respectively.

Identification and differential expression analysis of BR-induced miRNAs

Known miRNAs in the sequenced data were identified using BLASTN function in miRBase database using *A. thaliana* genome as reference. A total of 229 known miRNAs were identified in all the four libraries, and these belong to 102 known miRNA families. As shown in the Venn diagram, a total of 171 miRNAs identified were observed to be common in all the four libraries, and this accounted for 74.7% of the total miRNAs identified in all the four samples (Fig. 4a). Whereas five miRNAs (2.2%) were identified as BR-R specific, two miRNAs each (0.9%) were identified in BR-S and No-BR-R-specific libraries while four miRNAs (1.7%) were identified in

Fig. 1 Analysis of seed germination and seedling growth in *A. thaliana* in response to BR treatment. **a** Images of 2-day-old seeds germinated on MS media supplemented with different concentrations of BR. Effects on primary root growth and root hair formation were analysed. **b** Five-day-old seedlings grown on MS media and transferred to fresh media plates supplemented with different concentrations of BR. Black dots were marked at the position showing tips of elongated primary root at the end of 5 days immediately after transferring on BR-supplemented MS plates. Morphological and biochemical analysis was performed on these seedlings after 7 days of growth on BR-supplemented media. **c** Bar graphs showing changes in fresh weight of seedlings and activity of superoxide dismutase in seedlings. Statistical analysis using one-way ANOVA followed by Dunnett's *t* test was performed and expressed as mean ± SD. ***$P > 0.001$, **$P > 0.01$, *$P > 0.05$; ns not significant. Asterisks indicate significant differences in fresh weight biomass between control (0 nM) and BR-treated seedlings
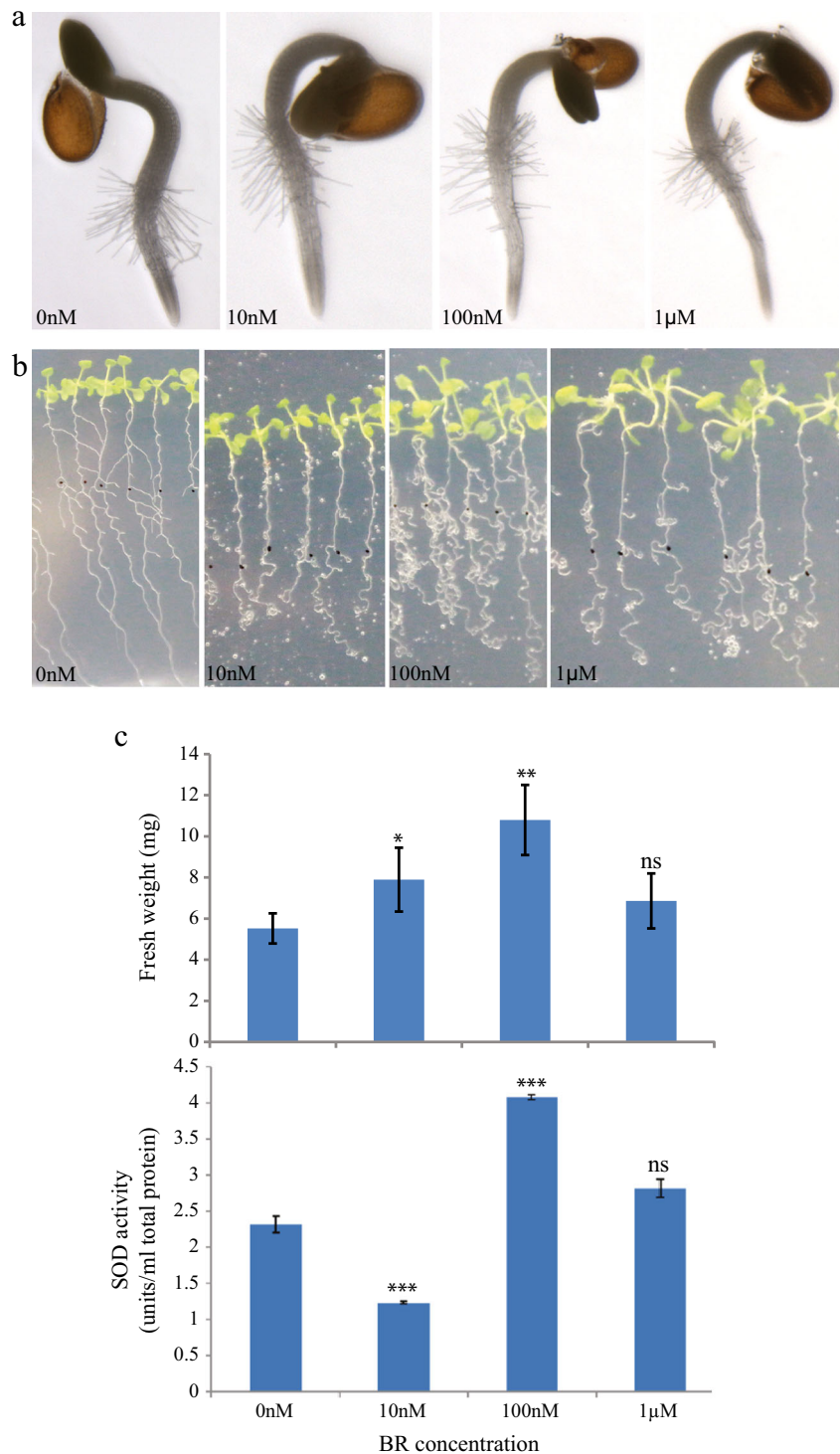
No-BR-S sample. In order to identify which of the known miRNA species were expressed in response to BR treatment, differential expression of miRNAs was analysed using normalized reads in all the four samples. Scatter plots by transforming the data onto M (log ratio) and A (mean average) scales and then plotting the values to generate MA plots showed differential and baseline-expression patterns of miRNAs in root and shoot tissues where each dot represents individual miRNA (Fig. 4b, c). MicroRNAs showing changes in expression levels ≥ 1.0-fold were considered as differentially expressed while those with expression levels below 1-fold were considered as being in baseline-expression zone. Cluster heatmap of differentially expressed known miRNAs was generated
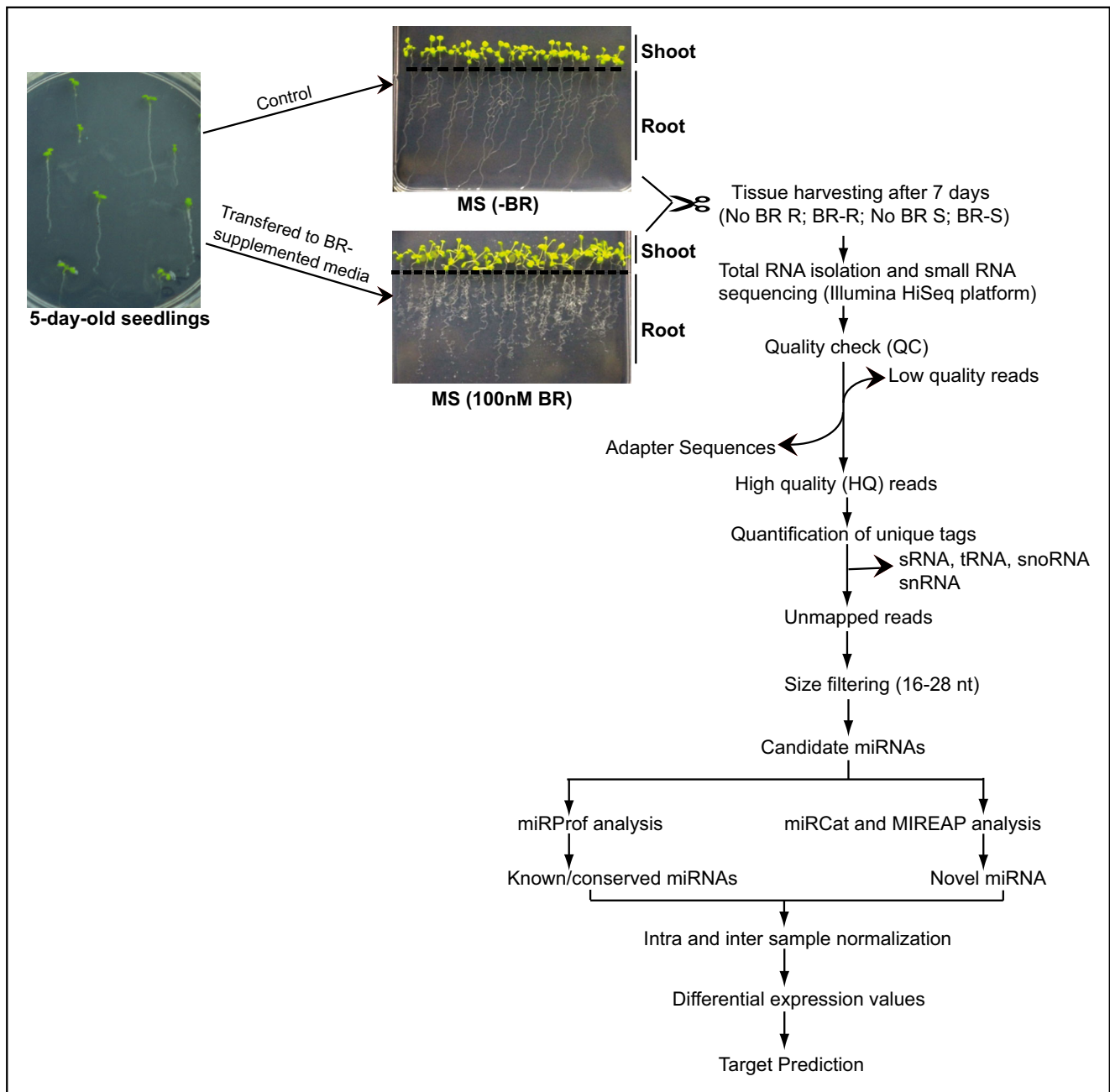
Fig. 2 Schematic representation of tissue sampling process and flow chart depicting steps followed in the study from tissue harvesting to analysis of small RNA population using NGS platform

using absolute expression values of treated and control samples. The miRNA families miR169 (miR169d, miR169e, miR169f, miR169g), miR156 (miR156a, miR156g), miR172 and those belonging to unknown families, such as miR5648, miR5653, miR5654 and miR6563, were observed to be deferentially expressed in response to BR treatment (Table 3; Fig. 4d). For example, miR172d-3p, miR172c, miR829p-3p, miR846-5p, miR5666 and miR5643a were observed to be most differentially expressed between root and shoot samples (Fig. 4d marked as ##), while miR169g-5p, miR169f-5p,

miR169e and miR169d (marked as *# in Fig. 4d) showed decreased expression upon BR treatment in roots.

Out of 229 known miRNAs belonging to 102 families identified in this study, 48 miRNA families showed differential expression in response to BR treatment in root and shoot tissues (Table 3). The miR5663-3p belonging to an unknown family with 7.924813 fold change showed highest expression in root, followed by miR8121 (7.857981 fold change), miR830-3p belonging to miR830 family (7.667606 fold change), miR850 (7.636625 fold change), miR5637 (7.554589 fold change)

Table 1 Summary of data obtained for No BR-R, No BR-S, BR-R and BR-S sequenced libraries

Type	No-BR-R		No-BR-S		BR-R		BR-S	
	Counts	%	Counts	%	Counts	%	Counts	%
Total reads	24,816,172	100%	25,902,123	100%	25,210,454	100%	25,691,643	100%
High quality	24,786,485	99.88%	25,868,028	99.87%	25,182,528	99.89%	25,652,823	99.85%
Total no. of bases in high-quality reads	605,360,311	100%	652,417,477	100%	645,461,180	100%	667,847,519	100%
Total no. of high-quality bases in high-quality reads	599,907,077	99.10%	646,761,747	99.13%	639,580,615	99.09%	661,430,872	99.04%
Minimum read length	15		15		15		15	
Maximum read length	51		51		51		51	
Average read length	24.42		25.22		25.63		26.03	
Total tag length (bp)	47,964,576		65,015,911		61,614,163		53,662,516	
No. of unique tags	2,021,325		2,726,269		2,552,657		2,226,207	

of unknown family and miR859 (7.276124), respectively. On the other hand, miR169d, miR169e, miR169f-5p, miR169g-5p belonging to miR169 family with expression values of − 8.810572 showed strong downregulation in root, followed by miR156a-3p belonging to miR156 family (− 7.584963 fold change), miR173-3p (− 7.118941 fold change), miR447c-5p, belonging to miR447 family (− 7.118941 fold change), miR865-5p (− 6.882643 fold change) and miR833a-5p belonging to miR833 family (− 6.741467 fold change), respectively. The highest up-regulated miRNA in response to BR treatment in the shoot tissue was miR865-5p belonging to miR865 family with a fold change of 8.276124 with respect to control (No-BR-S). Whereas, miR835-5p belonging to miR835 family was observed to be significantly downregulated in shoots with a fold change − 8.076816 when compared with No-BR-S (Table 3).

Identification and expression analysis of BR-responsive novel miRNAs

After removal of known/conserved miRNAs, sequences were subjected to miRCat analysis to identify novel miRNAs expressed in response to BR treatment. Using the Arabidopsis genome database, a total of 27 novel miRNAs were identified (Table 4). Mature sequences of these novel miRNAs were observed to be 21–22 nucleotide in length, and 5′ nucleotide was observed to be 'U' in most of the miRNAs. Stem-loop sequences for each novel miRNAs were marked as per analysis with reference genome, and fold-back structures were predicted through Mfold software (Fig. S1). Majority of the identified novel miRNA candidates were observed to be located on chromosomes 1, 2 and 4, while only two miRNAs (miR-20 and miR-05) are present on

chromosome 3, while four miRNAs (miR-09, miR-10, miR-14 and miR-15) were observed to be localized on chromosome 5 (Fig. 5a).

Among the 27 novel miRNAs identified, 2 of them, i.e. miR-08 and miR-21, were observed to be expressed in all the four samples (Table S2; Fig. 5b). While, four miRNAs, i.e. miR-01, miR-03, miR-04 and miR-05, were observed to be BR-R specific and miR-12, miR-13, miR-14 and miR16 were identified as BR-S specific. On the other hand, miR-23, miR-24, miR-25, miR-26 and miR-27 were exclusively expressed in No-BR-S, while miR-17, miR-18, miR-19, miR-20 and miR-22 were observed to be No-BR-R specific (Table S2; Fig. 5b). Differential expression analysis shows seven miRNAs miR-01, miR-02, miR-03, miR-04, miR-05, miR-07 and miR-10 in roots and six miRNAs miR-02, miR-09, miR-12, miR-13, miR-14 and miR-16 in shoots to be significantly up-regulated (Fig. 5c; Table 4). Similarly, six putative novel miRNAs, namely miR-06, miR-17, miR-18, miR-19, miR-20 and miR-22, in roots and six miRNAs miR-07, miR-23, miR-24, miR-25, miR-26 and miR-27 in shoots were observed to be significantly downregulated (Fig. 5c; Table 4). The most significantly upregulated miRNAs in roots are miR-10 (10.943 fold change) followed by miR-01 (10.586 fold change), miR-07 (9.8602 fold change), miR-04 (7.5554 fold change) and miR-03 (6.7898 fold change), while in shoots, these are miR-02 (14.628 fold change), miR-16 (9.4429 fold change), miR-09 (8.7345 fold change) and miR-13 (7.5970 fold change) (Table 4). On the other hand, significantly downregulated miRNAs in roots are miR-20 (− 14.618 fold change), miR-17 (− 14.053 fold change) and miR-18 (− 13.105 fold change), while in shoots, these are miR-26 (− 11.800 fold change), miR-24 (− 8.8600 fold change) and miR-07 (− 7.826 fold change), respectively (Table 4). MA plot for root and

a

b
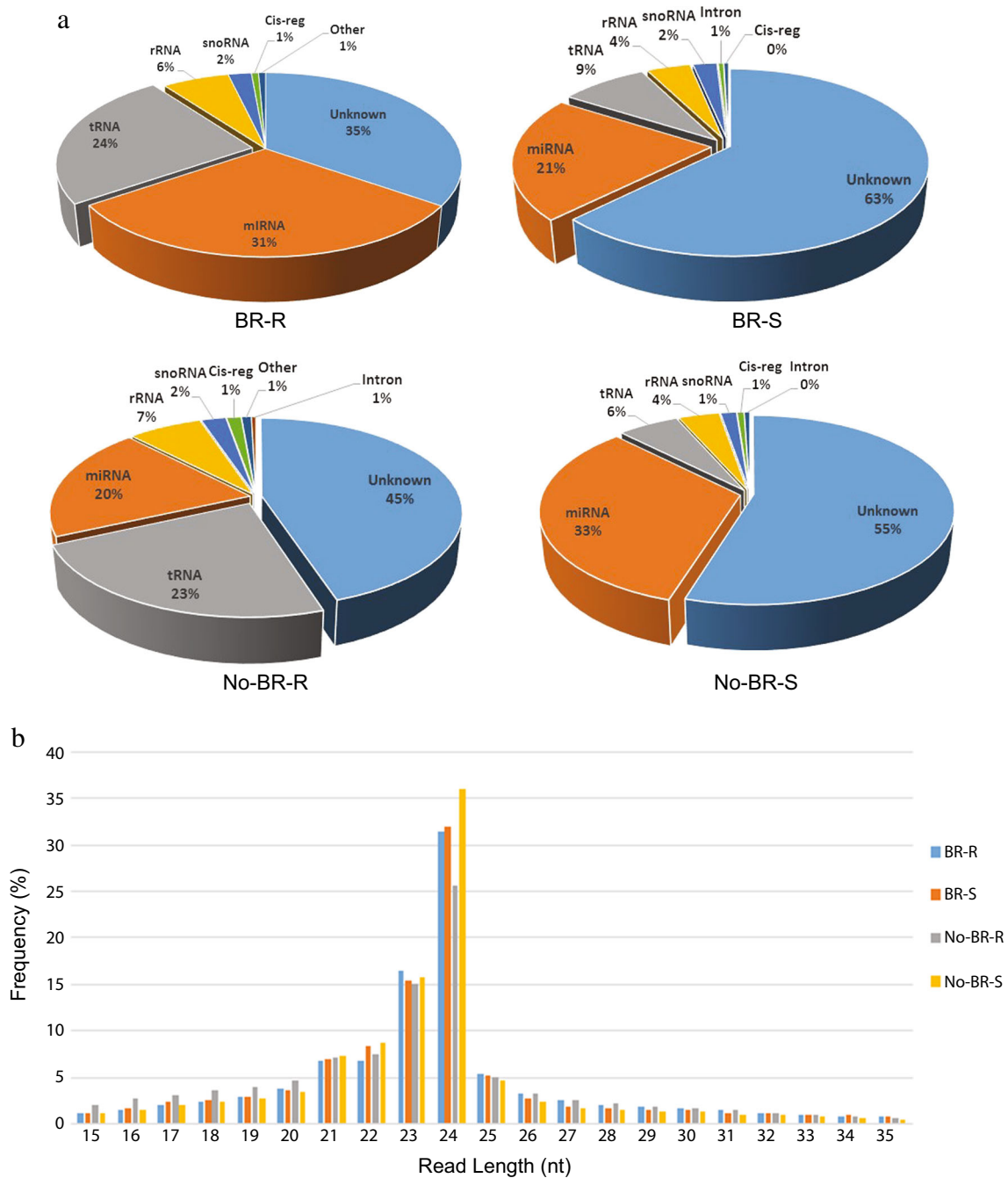
Fig. 3 **a** Percent distribution of small RNAs in the libraries. **b** Length distribution of small RNAs in the libraries where small RNAs of 24 nt were showing in maximum

Table 2 Small RNAs and mean frequencies in No BR-R, No BR-S, BR-R and BR-S libraries	Class	Unique sRNAs	Total sRNAs	Mean frequency
	Total sRNAs	435,146	51,715,668	118.85
	No-BR-R, No-BR-S, BR-R and BR-S common	35,348	10,699,110	302.68
	No-BR-R specific	88,193	128,024	1.45
	No-BR-S specific	79,197	121,095	1.53
	BR-R specific	82,228	112,732	1.37
	BR-S specific	6268	122,206	19.50

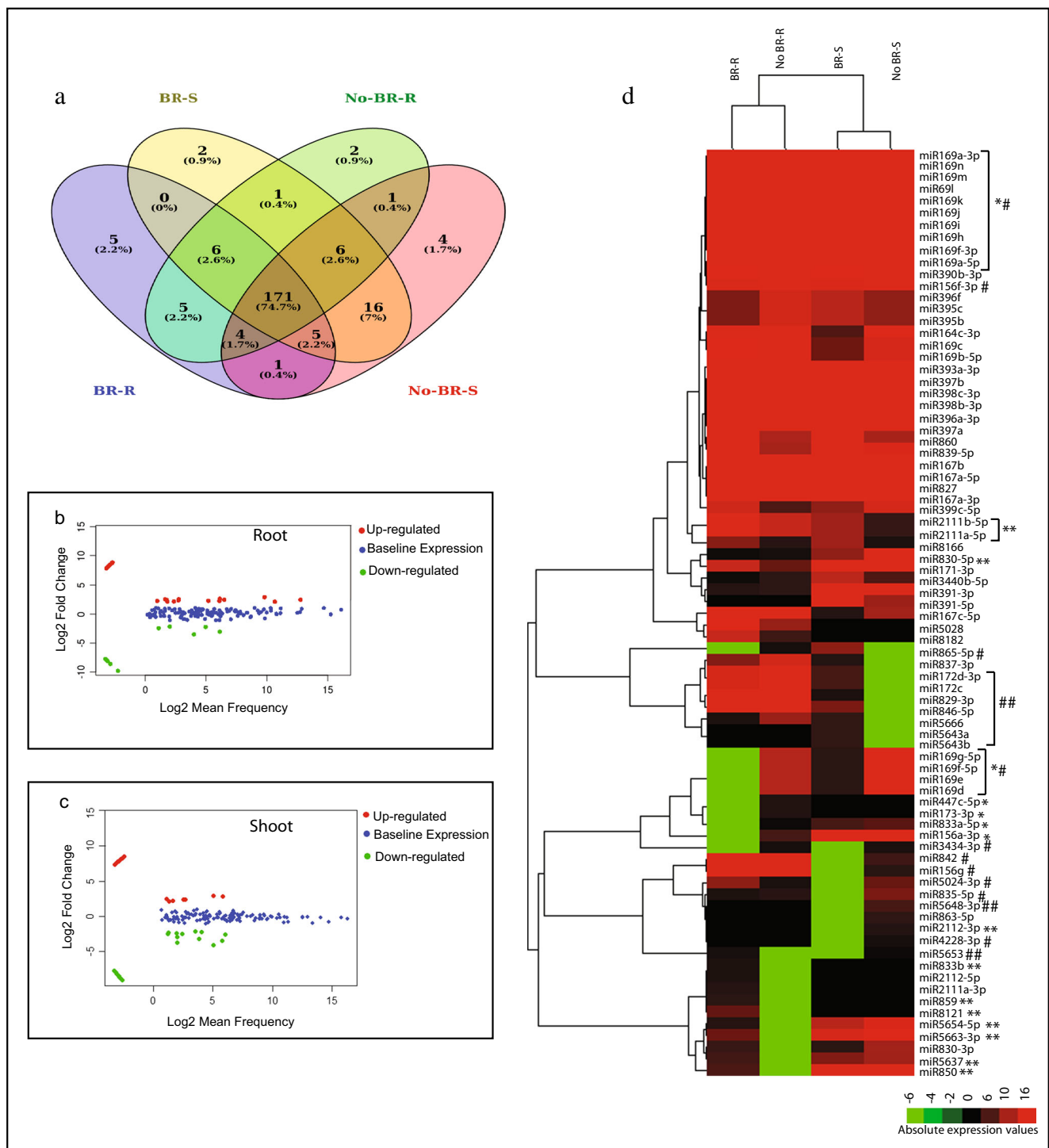**Fig. 4** Distribution and expression pattern of known miRNAs in different tissue libraries. **a** Venn diagram showing distribution of known miRNAs in BR-S, No-BR-S, BR-R and No-BR-R libraries. **b** MA plot of differentially expressed known miRNAs in roots. **c** MA plot of differentially expressed known miRNAs in shoot samples. **d** Heat map of differentially expressed known miRNAs in all four libraries. Asterisk indicates downregulated miRNAs in BR-R; double asterisk indicates upregulated miRNAs in BR-R; number sign indicates downregulated miRNAs in BR-S; double number sign indicated upregulated miRNAs in BR-S; asterisk and number sign indicates downregulated miRNAs in both roots and shoots in response to exogenous BR (BR-R and BR-S)

shoot samples was generated separately by using the log$_2$ fold change values, and those showing ≥1 fold change

were categorized as differentially expressed while those with < 1 fold change as baseline-expressed (Fig. 5d, e).

Table 3 Differentially expressed BR-responsive known miRNAs identified in *A. thaliana* root and shoot samples

BR-R vs. No-BR-R

Family	miRNA name	BR-R		No-BR-R		Fold change ($\log_2$ treatment/control)	Involvement in hormone signalling
		Counts	Normalized*	Counts	Normalized*		
miR169	miR169a-3p	62	6.86	355	37.92	− 2.46668	AUX and ABA (Ni et al. 2013)
	miR169a-5p	182	20.69	442	47.22	− 1.19046	
	miR169d	0	0	42	4.49	− 8.810572	
	miR169e	0	0	42	4.49	− 8.810572	
	miR169f-5p	0	0	42	4.49	− 8.810572	
	miR169f-3p	436	48.24	909	97.01	− 1.00924	
	miR169g-5p	0	0	42	4.49	− 8.810572	
	miR169h	314	34.74	1314	140.26	− 2.01343	
	miR169i	314	34.74	1314	140.26	− 2.01343	
	miR169j	314	34.74	1314	140.26	− 2.01343	
	miR169k	314	34.74	1314	140.26	− 2.01343	
	miR169l	314	34.74	1314	140.26	− 2.01343	
	miR169m	314	34.74	1314	140.26	− 2.01343	
	miR169n	314	34.74	1314	140.26	− 2.01343	
miR156	miR156a-3p	0	0	18	1.92	− 7.584963	GA signalling (Yu et al. 2012)
miR173	miR173-3p	0	0	13	1.39	− 7.118941	Not reported
Unknown	miR5666	12	1.33	33	3.53	− 1.40824	
Unknown	miR3434-3p	0	0	11	1.18	− 6.882643	
miR447	miR447c-5p	0	0	13	1.39	− 7.118941	
miR395	miR395b	25	2.77	56	5.98	− 1.11026	
	miR395c	25	2.77	56	5.98	− 1.11026	
	miR395f	25	2.77	56	5.98	− 1.11026	
miR833	miR833a-5p	0	0	10	1.07	− 6.741467	
miR837	miR837-3p	26	2.88	55	5.88	− 1.02974	
miR865	miR865-5p	0	0	11	1.18	− 6.882643	
Unknown	miR5640	357	39.5	750	80.12	− 1.02031	
miR167	miR167a-3p	15,706	1737.92	4296	458.92	1.920898	Auxin signalling (Wu et al. 2006)
	miR167a-5p	962	106.44	415	44.33	1.263685	
	miR167b	962	106.44	415	44.33	1.263685	
miR397	miR397a	1132	125.25	368	39.31	1.671842	Not reported
	miR397b	21,440	2372.16	10,130	1082.14	1.132314	
miR830	miR830-3p	16	1.77	0	0	7.667606	
miR833	miR833b	12	1.33	0	0	7.055282	
miR839	miR839-5p	105	11.62	37	3.95	1.556686	
Unknown	miR8182	48	5.31	17	1.82	1.544773	
	miR5637	17	1.88	0	0	7.554589	
	miR5653	11	1.22	0	0	6.930737	
	miR5654-5p	13	1.44	0	0	7.169925	
	miR5663-3p	22	2.43	0	0	7.924813	
	miR8121	21	2.32	0	0	7.857981	
miR398	miR398b	104,838	11,600	38,874	4152.73	1.481993	
	miR398c	104,838	11,600	38,874	4152.73	1.481993	
miR399	miR399c	51	5.64	19	2.03	1.474215	
miR827	miR827	1355	149.92	509	54.37	1.46331	
miR850	miR850	18	1.99	0	0	7.636625	
miR859	miR859	14	1.55	0	0	7.276124	
miR860	miR860	97	10.73	39	4.17	1.363531	
miR396	miR396a	521	57.64	219	23.39	1.301178	GA, CK and ABA pathways (Hewezi et al. 2012)
Unknown	miR5024	28	3.1	12	1.28	1.276124	Not reported
	miR5028	70	7.74	32	3.42	1.178337	
miR171	miR171a	48	5.31	22	2.35	1.176051	GA signalling (Ma et al. 2014)
miR158	miR158b	6608	721.12	3287	351.13	1.058103	Not reported
miR159	miR159b-5p	24	2.66	12	1.128	1.055282	GA, ABA and ET pathway (Curaba et al. 2014)
	miR159b-3p	16,390	1813.42	8352	892.2	1.023274	
miR165	miR165a	340,839	37,700	170,428	18,200	1.050626	Not reported
	miR165b	340,839	37,700	170,428	18,200	1.050626	
miR857	miR857	226	25.01	114	12.18	1.037991	
miR2111	miR2111a-3p	13	1.44	0	0	7.169925	
miR2112	miR2112-5p	12	1.33	0	0	7.055282	

Table 3 (continued)

BR-R vs. No-BR-R

Family	miRNA name	BR-R		No-BR-R		Fold change (log$_2$ treatment/control)	Involvement in hormone signalling
		Counts	Normalized*	Counts	Normalized*		

BR-S vs. No-BR-S

Family	miRNA name	BR-S Counts	Normalized*	No-BR-S Counts	Normalized*	Fold change (log$_2$ treatment/control)	Involvement in hormone signalling
miR169	miR169f-3p	88	11.36	867	93.69	−3.04393	AUX and ABA (Ni et al. 2013)
	miR169d	12	1.55	97	1.48	−2.7573	
	miR169e	12	1.55	97	1.48	−2.7573	
	miR169f-5p	12	1.55	97	1.48	−2.7573	
	miR169g-5p	12	1.55	97	1.48	−2.7573	
	miR169h	179	23.1	1200	129.68	−2.48899	
	miR169i	179	23.1	1200	129.68	−2.48899	
	miR169j	179	23.1	1200	129.68	−2.48899	
	miR169k	179	23.1	1200	129.68	−2.48899	
	miR169l	179	23.1	1200	129.68	−2.48899	
	miR169m	179	23.1	1200	129.68	−2.48899	
	miR169n	179	23.1	1200	129.68	−2.48899	
	miR169a-5p	291	37.56	1050	113.47	−1.59504	
	miR169b-5p	19	2.45	58	6.27	−1.35568	
	miR169c	19	2.45	58	6.27	−1.35568	
	miR169a-3p	85	1097	230	24.85	−1.17968	
miR156	miR156f-3p	52	6.71	284	30.69	−2.19338	GA signalling (Yu et al. 2012)
	miR156g	0	0	14	1.51	−7.238405	
miR164	miR164c-3p	16	2.07	73	7.89	−1.93039	AUX/CK signalling (Sobkowiak et al. 2012)
miR830	miR830-5p	25	3.23	83	8.97	−1.47357	Not reported
	miR830-3p	12	1.55	36	3.89	−1.3275	
miR167	miR167c-5p	11	1.42	36	3.89	−1.45388	Auxin signalling (Wu et al. 2006)
miR390	miR390b-3p	61	7.87	161	17.4	−1.14465	AUX signalling pathway (Williams et al. 2005)
miR835	miR835-5p	0	0	25	2.7	−8.076816	Not reported
miR842	miR842	0	0	17	1.84	−7.523562	
miR847	miR847	116	14.97	282	30.47	−1.02532	
miR863	miR863-5p	0	0	15	1.62	−7.33985	
Unknown	miR2112-3p	0	0	13	1.4	−7.129283	
	miR3434-3p	0	0	12	1.3	−7.022368	
	miR4228-3p	0	0	11	1.19	−6.894818	
	miR5024-3p	0	0	22	2.38	−7.894818	
	miR5648-3p	0	0	18	1.95	−7.60733	
	miR5653	0	0	10	1.08	−6.745888	
miR172	miR172c	15	1.94	0	0	7.599913	GA signalling (Yu et al. 2010)
	miR172d-3p	15	1.94	0	0	7.599913	
miR393	miR393a-3p	496	64.02	158	17.07	1.90706	ABA-to-AUX signal (Sunkar and Zhu 2004)
miR391	miR391-3p	837	108.03	282	30.47	1.825971	Not reported
	miR391-5p	75	9.68	33	3.57	1.439083	
Unknown	miR8166	29	3.74	12	1.3	1.524527	
	miR5666	13	1.68	0	0	7.392317	
miR829	miR829-3p	10	1.29	0	0	7.011227	
miR837	miR837-3p	11	1.42	0	0	7.149747	
miR846	miR846-5p	21	2.71	0	0	8.082149	
miR860	miR860	81	10.45	37	4	1.385431	
miR865	miR865-5p	24	3.1	0	0	8.276124	
miR3440	miR3440b-5p	37	4.78	19	2.05	1.221387	
miR2111	miR2111a-5p	29	3.74	16	1.73	1.112266	
	miR2111b-5p	29	3.74	16	1.73	1.112266	
miR5643	miR5643a	13	1.68	0	0	7.392317	
	miR5643b	13	1.68	0	0	7.392317	

Fold change was calculated using the formula, fold change = log$_2$ (treatment / control) (Marsit et al. 2006). Significance was calculated as fold change log$_2$ > 1 or log$_2$ < − 1 and P value < 0.01. Only significantly expressed miRNAs are explained in the table

*miRNA expressions were normalized as read per million (RPM). If miRNA expression measured zero, normalized expression value was taken as 0.01 according to Murakami et al. (2006)

Table 4 Differentially expressed BR-responsive novel miRNAs in *A. thaliana* root and shoot samples

BR-R vs. No BR-R

| miRNA	Sequence (5′ to 3′)	BR-R		No-BR-R		Fold change (log$_2$ FC treatment/control)
		Count	Normalized*	Count	Normalized*	
miR-01	AAAATTGGGGGTGTAGCTCATA	139	15.38072998	0	0	10.586
miR-02	AAGATCCGGACTACAACAAAGC	93	10.29070423	42	4.4870825	1.1974
miR-03	AGCGAACCGACCGTCAGATAGG	10	1.106527337	0	0	6.7898
miR-04	AGCGGTTGTTAGCGATTGGCAC	17	1.881096472	0	0	7.5554
miR-05	CTCAACTCAATCCATGAACCCT	17	1.881096472	0	0	7.5554
miR-06	GCAAGTTGACCTTGGCTCTG	144	15.93399365	367	39.208554	− 1.299
miR-07	GTTCCTCTGAGCACTTCATT	84	9.294829628	0	0	9.8602
miR-10	TGTCAGTATAAATCTTTGATC	178	19.69618659	0	0	10.943
miR-17	ATGCCTGGCTCCCTGTATGCC	0	0	1592	170.08179	− 14.053
miR-18	ATGGACCGTCCAACTTGGCCC	0	0	825	88.139122	− 13.105
miR-19	GTCAAAGTTGGTATTGCAAACC	0	0	19	2.0298706	− 7.6652
miR-20	TCTGTGATTCTCTGTGTAAGC	0	0	2354	251.49029	− 14.618
miR-22	TTTGTACAACATTTTTAGTGT	0	0	16	1.7093647	− 7.4173
BR-S vs. No-BR-S						
miRNA	Sequence (5′ to 3′)	BR-S Count	Normalized*	No-BR-S Count	Normalized*	Fold change (log$_2$ FC treatment/control)
miiR-02	AAGATCCGGACTACAACAAAGC	1963	253.3731993	0	0	14.628
miR-07	GTTCCTCTGAGCACTTCATT	0	0	21	2.2692897	− 7.826
miR-09	TGGGACTGCCTAAGCTAAAGG	33	4.259457756	0	0	8.7345
miR-12	GACGGACGAGCTCGAGGTAGCT	27	3.485010892	0	0	8.4450
miR-13	GGGAATTCGTCGGCGATTTTGA	15	1.936117162	0	0	7.5970
miR-14	TCCGTCTTTATATTCATCATC	24	3.097787459	0	0	8.2750
miR-16	TGCGCAACTCTATATTTCCTC	54	6.970021783	0	0	9.4429
miR-23	CGAGTAAAGCTGTCGAGTGGC	0	0	13	1.4047984	− 7.1342
miR-24	GATTCGTACACTTAGATTGT	0	0	43	4.6466410	− 8.8600
miR-25	GTAACGTCTGGCTGAGTTATA	0	0	15	1.6209212	− 7.3406
miR-26	TCAGCTGAGCTTTCTCGTCATC	0	0	330	35.666026	− 11.800
miR-27	TTCACAGTAGATAGATATCGAC	0	0	16	1.7289827	− 7.4337

Fold change was calculated using the formula, fold change = log$_2$ (treatment / control) (Marsit et al. 2006). Significance was calculated as fold change log$_2$ > 1 or log$_2$ < − 1 and *P* value < 0.01. Only significantly expressed miRNAs are explained in the table

*miRNA expressions were normalized as read per million (RPM). If miRNA expression measured zero, normalized expression value was taken as 0.01 according to Murakami et al. (2006)

Target prediction of differentially expressed miRNAs and their functional categorization

Target genes of differentially expressed miRNAs were predicted on the basis of psRNATarget analysis using *A. thaliana* as genome reference. Target genes of most of the known and conserved miRNAs were similar to known targets identified in different studies (Table S3). While, among the novel miRNAs identified in this study, target genes for only six could be predicted (Table 5). Among these, auxin-responsive factors (ARFs) were predicted to

Fig. 5 Distribution and expression pattern of novel miRNAs in the root and shoot BR treated and untreated tissue libraries. **a** Distribution of novel miRNA precursor genes on different chromosomes of *A. thaliana*. **b** Distribution of novel miRNAs among the four libraries. **c** Heat map of differentially expressed novel miRNAs. High and low expression values are shown in colour code with red indicating strong expression while green indicating weak expression. **d** MA plot of differentially expressed novel miRNAs in roots. **e** MA plot of differentially expressed novel miRNAs in shoot samples. Single asterisk indicates upregulated miRNAs in BR-R; double asterisk indicates upregulated miRNAs in BR-S; triple asterisk indicates downregulated miRNAs in BR-R; quadruple asterisk indicates downregulated miRNAs in BR-S
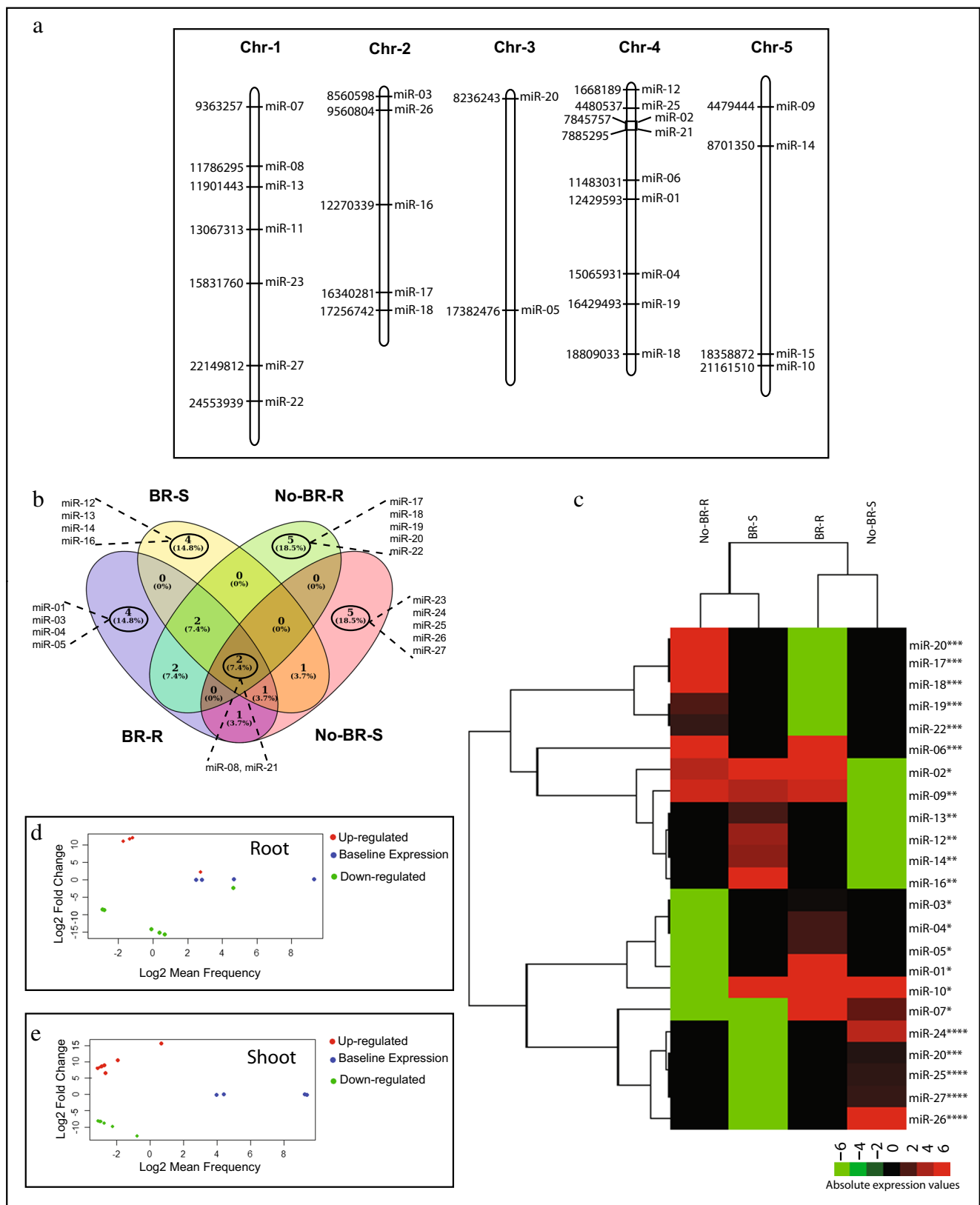
be direct targets of miR-17 that shows positive BR-responsive expression in shoots and is downregulated in roots (Fig. 5; Table 5).

GO analysis of all the differentially expressed miRNAs was performed. This analysis showed that predicted target genes are mainly involved in transcriptional processes and regulation.

Table 5 Potential targets identified for differentially expressed novel miRNAs

Name of miRNAs	Target genes	Regulation	Function
miR-12	Transposable element gene	Cleavage	Unknown
miR-14	Glycosyl hydrolase family protein 43	Cleavage	Involved in intracellular processes (Mewis et al. 2016)
miR-17	ARF10 (auxin response factor 10)	Cleavage	Auxin signalling (Mallory et al. 2005)
	ARF16 (auxin response factor 16)		
	ARF17 (auxin response factor 17)		
miR-20	Protein of unknown function, DUF538	Cleavage	Unknown
miR-23	Transposable element gene	Cleavage	Unknown
miR-27	Sec14p-like phosphatidylinositol transfer family protein	Cleavage	Involved in intracellular processes (Huang et al. 2016)

With respect to cellular component, the targets were mainly functional in the nucleus and these were largely involved in metabolic pathways (Fig. 6). This analysis therefore showed that target genes of BR-induced miRNAs were mainly involved in distinct molecular, cellular and metabolic processes in plants.

Expression validation of BR-responsive miRNAs and their target genes

To validate differential expression patterns of BR-responsive miRNAs, six known and novel miRNAs were randomly selected and their expression patterns was analysed using stem-loop RT-qPCR. The result of stem-loop RT-qPCR was compared with abundance of each selected miRNAs in the small RNA libraries

(Fig. 7a). It was observed that expression patterns for miR169a, miR167a, miR156f, miR393a, miR-01 and miR-16 correlated with results obtained in the small RNA sequencing study.

We also validated expression of three genes *AthNFYA3*, *AthARF10* and *AthSPL2* that are known targets of miR169a, miR167a and miR156f identified as BR-responsive in this study (Fig. 7b). Comparison of expression patterns of miRNAs with their respective targets showed that steady-state levels of miRNA transcripts were inversely related to expression patterns of their respective target genes (Fig. 7a, b). For instance, expression of *AthNFYA3* and *AthSPL2* was observed to be upregulated in contrast to expression of their targeting miRNAs miR169a and miR156f that were downregulated in both root and shoot samples. Similarly, *AthARF10*
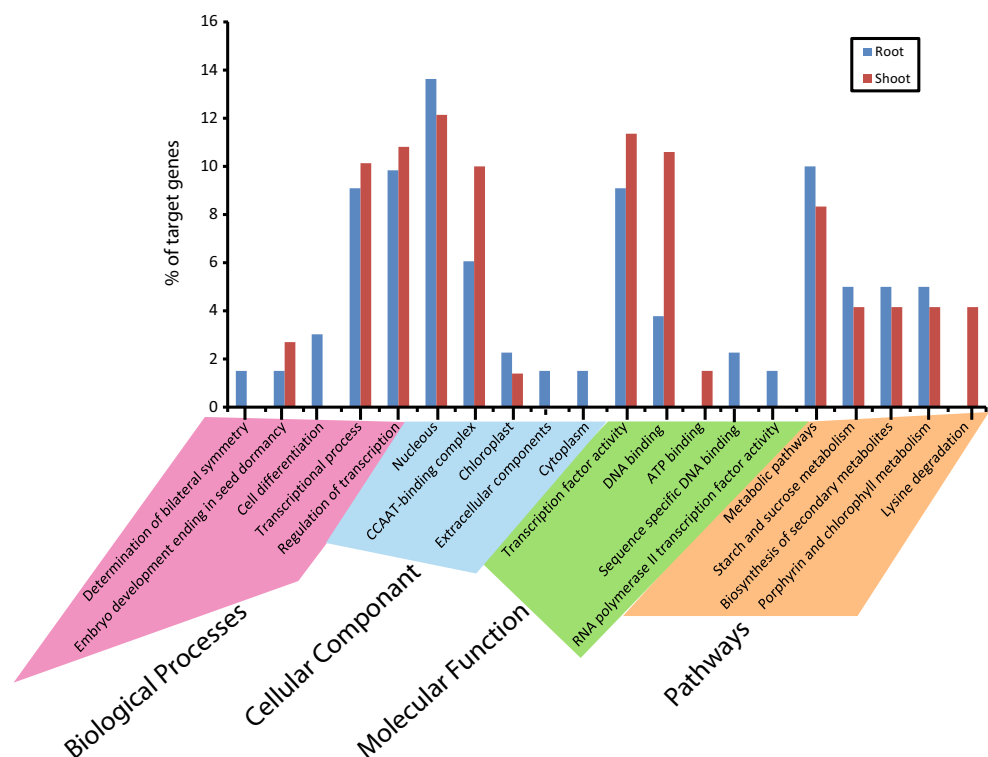
Fig. 6 Gene ontology (GO) analysis of target genes of differentially expressed miRNAs in response to exogenous BR treatment to root and shoot samples

Fig. 7 Expression validation using stem-loop RT-qPCR and RT-qPCR. Expression validation of selected BR-responsive miRNAs and their target genes. **a** Relative expression of known (miR169a, miR167a, miR156f and miR393a) and novel miRNAs (miR-01 and miR-16) as determined by stem-loop RT-qPCR. **b** Relative transcript abundance of selected target genes of conserved miRNAs, miR169a, miR167a and miR156f identified as BR-responsive in this study by RT-qPCR. Statistical analysis using one-way ANOVA followed by Dunnett's t test is expressed as mean ± SD where ***$P > 0.001$, **$P > 0.01$ and *$P > 0.05$. Asterisks indicate significant differences in relative expression levels of miRNAs between control and BR-treated tissue samples
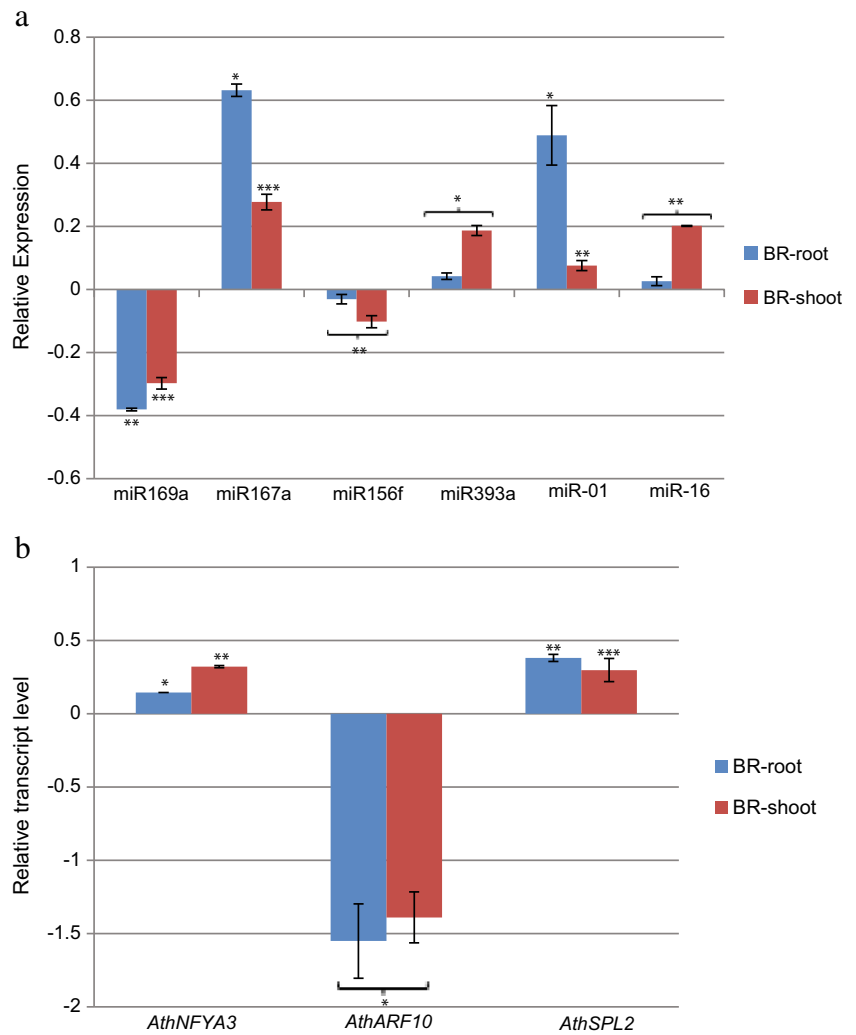
that is known to be targeted by miR167 members was observed to be downregulated in contrast to miR167a expression that showed increased expression in response to BR.

Discussion

MicroRNAs play an important role in regulating gene expression at post-transcriptional levels. In the last few years, deep sequencing technology has been used to identify miRNAs in plant genomes. By employing this technique, researchers have been able to identify and characterize miRNAs involved in specific signalling pathways. To date, many miRNAs have been functionally characterized and demonstrated to be involved in various mechanisms like phytohormone signalling, biotic and abiotic stresses, plant development and nutrient signalling pathways (Pant et al. 2009; Akpinar et al. 2015; Jian et al. 2016). Among these, phytohormones such as brassinosteroids have been the focus of many studies because of their diverse roles in plant development and stress-adaptive

responses. However, there are still many gaps in our understanding related to brassinosteroid research that needs attention.

The aim of this study was to identify brassinosteroid-responsive miRNAs expressed in tissue-specific manner in the model plant *A. thaliana*. To achieve this, small RNA sequencing was carried out using control (No-BR) and BR-supplemented root and shoot samples. Saliently, brassinosteroid treatment was observed to significantly affect miRNA population as evident from dominance of 24-nt small RNA molecules in filtered libraries. A total of 229 known and 27 novel miRNAs have been identified that were observed to be differentially expressed in response to BR. The 229 known miRNAs belonging to 102 families included the most conserved miRNAs reported in *A. thaliana* (Sunkar and Zhu 2004). Moreover, conserved miRNAs were observed to be more abundant in comparison to non-conserved miRNAs. The most abundant miRNA family in both root and shoot libraries was observed to be miR169. Interestingly, all members of miR169 were observed to be downregulated in root and shoot tissues in response to BR application. Previous
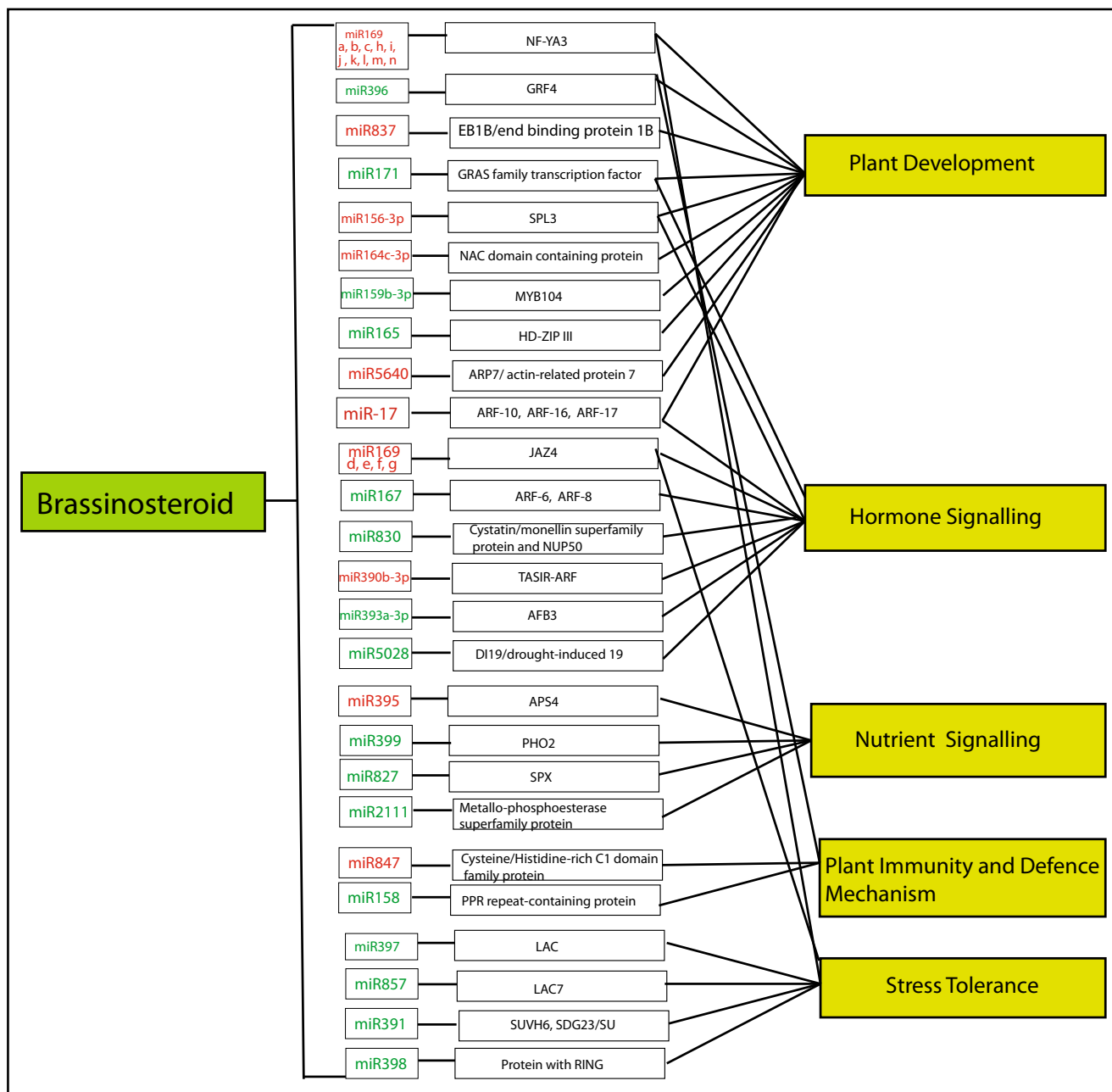
Fig. 8 Possible roles of conserved and novel BR-induced miRNAs in different biological processes in plants based on GO study. Down regulated BR-responsive miRNAs are shown in red, while those shown in green represent upregulated BR-responsive miRNAs identified in this study

studies have shown that miR169 is mainly involved in abiotic stress and AUX/ABA signalling (Ni et al. 2013). These results therefore suggest miRNA-based crosstalk between brassinosteroid and auxin signalling pathways. Auxins are known to be key players in plant development process (Ubeda-Tomás et al. 2012). Brassinosteroids are also known to regulate many aspects of plant development and stress responses (Sahni et al. 2016). However, clear genetic evidence of their integrated effects on plant growth and stress tolerance is still lacking.

Characterization and comparative profiling of the miRNAs between BR and No-BR samples have provided insight into the possible involvement of miRNAs in BR signalling. Differential expression analysis showed 59 miRNAs in root library while only 50 in shoots to be differentially expressed. This indicates that root microRNA biome is more sensitive to BR treatment than shoot microRNA population. Plants exposed to BR showed increase in miRNA population by 8% in comparison to No-BR root samples. On the other hand, a

12% decrease of miRNAs in BR-exposed shoot tissues was observed in comparison to No-BR shoot samples. Previous studies have shown that proliferation and differentiation root cells are highly influenced by interactive role of auxins and brassinosteroids (Chaiwanon and Wang 2015). Many biochemical assays have also suggested a synergistic interaction between auxins and BRs (Goda et al. 2004).

Relationship between miRNAs and its target genes has been the focus of miRNA-associated genetic regulation. Prediction of target genes of differentially expressed miRNAs provides valuable information about miRNA-mediated biological processes interlinked with phytohormone signalling pathways in general. Target gene annotations of potential miRNAs have provided insight into gene regulation in response to BR exposure. The precursor sequence of novel miRNAs identified in this study showed fold-back hairpin structures and as expected had low free energy (− 30 to − 57 kcal/mol). A majority of miRNAs are known to have lower free energies as compared to tRNAs and rRNAs (Bonnet et al. 2004). In silico analysis showed that some miRNAs may be involved in targeting genes affecting hormone signalling and metabolism. GO analysis showed that BR-responsive miRNAs could also be involved in regulating genes affecting plant development, hormone signalling and stress responses (Fig. 8). Validation of expression of three of the genes *AthARF10*, *AthNFYA3* and *AthSPL2* that are targets of miR167, miR169 and miR156 and identified as BR-responsive in this study showed that expression of these genes was inversely related to levels of targeting miRNAs. ARFs mediate auxin signalling and regulate plant development; *AthNFYA3* is known to be involved in abiotic stress responses, while *AthSPL2* affects different aspects of plant development as well (Shikata et al. 2009; Leyva-Gonza'lez et al. 2012; Nicholas and Naden 2017). This observation suggests a direct role of BR in affecting miRNA-regulated transcript levels of genes regulating plant development and responses to various stress conditions.

Many recent studies support the role of BR and miRNAs in modulating plant development and metabolic processes in responses to environmental cues (Jay et al. 2010; Sharma et al. 2012). Differential expression of miRNAs in response to drought/dehydration stress has been documented in plants such as barley, *Brachypodium distachyon* and wild emmer wheat (Budak and Akpinar 2011; Akpinar et al. 2015; Alptekin et al. 2017). These studies together with the results presented in this study support integrated roles of BR and miRNA-based post-transcriptional gene regulatory mechanisms that can be further investigated for better understanding of molecular basis of BR-mediated stress tolerance in plants.

Conclusions

- This study provides a comprehensive compilation of tissue-specific BR-responsive miRNomes in *A. thaliana* seedlings.
- A number of conserved miRNAs were observed to be differentially expressed in roots and shoots in response to exogenously supplied 24-epibrassinolide.
- Twenty-seven novel miRNAs were identified that show tissue-specific differential expression in a BR-responsive manner. These novel miRNAs did not show any sequence conservation with known miRNAs in databases. Auxin response factors were predicted as target genes for one of the novel miRNA, miR-17.
- This work lays the foundation for further investigation into understanding the underlying molecular mechanisms of BR-mediated stress tolerance by post-transcriptional gene regulation.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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