# MicroRNA Regulation of Abiotic Stress Response in *7B-1* Male-Sterile Tomato Mutant

Vahid Omidvar,* Irina Mohorianu, Tamas Dalmay, and Martin Fellner*

## Abstract

The *7B-1* tomato (*Solanum lycopersicum* L. 'Rutgers') is a male-sterile mutant with enhanced tolerance to abiotic stress in a blue-light (BL) specific manner compared with its wild-type (WT). This makes the *7B-1* a potential candidate for hybrid seed breeding and stress engineering. To identify small RNAs (sRNAs) linked to stress tolerance of *7B-1*, two sRNA libraries from BL-grown *7B-1* and WT seedlings treated simultaneously with abscisic acid (ABA) and mannitol were sequenced, and sRNA profiles were compared. Twenty nine families of known microRNAs (miRNAs) and 27 putative novel miRNAs were identified from the two libraries. MiR5300, miR5301, miR2916, and a novel miRNA denoted miR#C were upregulated, while miR159, miR166, miR472, miR482, and two novel miRNAs, miR#A and miR#D, were downregulated in stress-treated *7B-1* seedlings. MiRNA targets with potential roles in stress regulation were validated by rapid amplification of 5′ complementary DNA ends (5′-RACE) analysis. Expression of miR159, miR166, miR472, miR482, miR#A, and miR#D together with their targets were further investigated in response to ABA, mannitol, NaCl, and cold treatments and a strong negative correlation was observed between the levels of these miRNAs and expression of their targets. Only miR159 and miR166 responded to cold treatment. MiR#A and its target were regulated by ABA and mannitol as early as 0.5 h after the treatments, while other miRNAs and targets were regulated only after 2 h. This suggests a role in early response to stress for miR#A. Our data suggests that miR159, miR166, miR472, miR482, miR#A, and miR#D are likely to facilitate the BL-specific enhanced tolerance of *7B-1* to abiotic stress.

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TOMATO IS ONE of the major vegetable crops grown all over the world and because of the importance of male-sterility in developmental and molecular studies and in hybrid seed production, several male-sterile mutants have been identified and characterized (Emmanuel and Levy, 2002; Roy et al., 2012; Jeong et al., 2014). The *7B-1* mutant displays male-sterility under long days with shrunken stamens in which microsporogenesis breaks down before the meiosis in pollen mother cells, but under short days, it produces flowers with normal stamens and viable pollens (Sawhney, 1997; Sheoran et al., 2009). Compared with the WT, the *7B-1* is less sensitive to light-induced inhibition (i.e., de-etiolation) of hypocotyl growth and has an elevated level of endogenous ABA but less gibberellins, Indole-3-acetic acid, and cytokinins, and is hypersensitive to exogenous ABA (Fellner et al., 2001; Fellner and Sawhney, 2002; Bergougnoux et al., 2012). Seed germination in *7B-1* is more tolerant to exogenous ABA, osmotic, salt and low

V. Omidvar and M. Fellner, Group of Molecular Physiology, Lab. of Growth Regulators, Centre of the Region Haná for Biotechnological and Agricultural Research, Palacky Univ. & Institute of Experimental Botany ASCR, Olomouc, Šlechtitelů 27, CZ-78371 Olomouc, Czech Republic; I. Mohorianu and T. Dalmay, School of Computing Sciences, Univ. of East Anglia, Norwich, NR4 7TJ, UK, and School of Biological Sciences, Univ. of East Anglia, Norwich, NR4 7TJ, UK. Received 27 Feb. 2015. Accepted 27 May 2015. *Corresponding authors (vahid.omidvar@upol.cz; martin.fellner@upol.cz).

**Abbreviations:** 5′-RACE, rapid amplification of 5′ complementary DNA ends; ABA, abscisic acid; ABC, adenosine triphosphate-binding cassette; BL, blue light; miRNA, microRNA; phasiRNA, phased secondary small interfering RNA; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcription–polymerase chain reaction; siRNA, small interfering RNA; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA; sRNA, small RNA; tRNA, transfer RNA; WT, wild-type.

temperature stresses, specifically under BL (Fellner and Sawhney, 2002). Fellner and Sawhney (2002) demonstrated that there was a defect in BL perception in *7B-1*, which affected hormonal sensitivity and their endogenous levels. As a stress-tolerant male-sterile mutant, *7B-1* is a valuable germplasm for hybrid tomato breeding (Sawhney, 2004).

Different mechanisms of stress response contribute to stress resistance at different morphological, biochemical and molecular levels. Advancement of molecular biology research has shown that plants respond to stress not only at the messenger RNA (mRNA) or protein levels but also at the posttranscriptional level (Phillips et al., 2007; Covarrubias and Reyes, 2010). Recent studies suggest important roles of sRNAs, especially miRNAs in plant response and adaptation to biotic and abiotic stresses (reviewed by Kruszka et al., 2014; Rogers and Chen, 2013). sRNAs fall into two categories, miRNAs and short-interfering RNAs (siRNAs), which are distinguished by their biogenesis. Plant miRNAs are typically 21-nucleotide (nt)-long single-stranded RNAs, which are processed from typical stem-loop precursors by the Dicer-like 1 enzyme. MicroRNAs are incorporated into the RNA-induced silencing complex, guide it to target mRNAs, and negatively regulate their expression by cleavage or translational repression (Tang et al., 2003; Bartel, 2004; Axtell, 2013). Small interfering RNAs (siRNAs) are processed by members of the Dicer-like family from long double-stranded RNAs, which are derived from transcription of inverted repeat sequences, convergent transcription of sense–antisense gene pairs, or synthesis by RNA-dependent RNA polymerases (Dalmay et al., 2000; Axtell, 2013). Plant miRNAs can trigger the production of phased secondary siRNAs (phasiRNAs) from either noncoding (e.g., *TAS* loci) or protein-coding genes (e.g., *NBS-LRR* genes) (Zhai et al., 2011; Shivaprasad et al., 2012).

Abscisic acid-, drought-, salt- and cold-regulated miRNAs and their targets have been identified in several plants, including *Arabidopsis thaliana* (L.) Heynh., tomato, rice (*Oryza sativa* L.), maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), alfalfa (*Medicago sativa* L.), and soybean [*Glycine max* (L.) Merr.] using sRNA sequencing (Sunkar and Zhu, 2004; Zhao et al., 2007; Kantar et al., 2010; Kong et al., 2010; Trindade et al., 2010; Li et al., 2011; Cao et al., 2014). In tomato, 12 and 20 miRNAs were up- and downregulated, respectively, in response to cold stress (Cao et al., 2014). MiR159 level increased in *Arabidopsis* seedlings on exposure to ABA and drought. Overexpression of miR159, as well as miR159-resistant *MYB33* and *MYB101* in *Arabidopsis* resulted in ABA hypersensitivity, which suggests that ABA-induced accumulation of miR159 plays a role in homeostasis of *MYB33* and *MYB101* mRNA levels during hormone and stress responses (Reyes and Chua, 2007). MiR393 was strongly upregulated in *Arabidopsis* by ABA, dehydration, salt and cold treatments (Sunkar and Zhu, 2004). MiR417 negatively regulated germination of *Arabidopsis* seeds under salt stress (Jung and Kang, 2007). Liu et al. (2008) identified several stress-inducible miRNAs in *Arabidopsis* seedlings, among them miR168, miR171, and miR396 were upregulated by ABA, mannitol, and NaCl, miR167 by mannitol and NaCl, and miR156, miR159, and miR394 only by NaCl. MiR167, miR169, and miR319 were downregulated by ABA in rice (Liu et al., 2009), while miR169, miR171, and miR393 were upregulated under drought stress (Zhao et al., 2009; Jian et al., 2010). Zhou et al. (2010) identified 16 and 14 miRNAs, which were down- and upregulated, respectively, in rice in response to drought stress.

Most of the studies described above have just profiled the expression of miRNAs in response to stress treatments, linking miRNAs expression to stress response. However, very few have characterized the stress-related functions of these miRNAs and their targets. *Arabidopsis* seeds overexpressing miR160 exhibited ABA insensitivity and tolerance during germination (Liu et al., 2007). Overexpression of miR396 in *Arabidopsis* enhanced drought tolerance (Liu et al., 2009). MicroRNAs in tomato have been primarily characterized in fruit development and ripening process (Moxon et al., 2008a; Mohorianu et al., 2011; Karlova et al., 2013; Din and Barozai, 2014). Recently Cao et al. (2014) profiled several cold-induced miRNAs in tomato. Jin et al. (2012) identified three miRNAs, which were differentially expressed in tomato in response to the *Botrytis cinerea* pathogen. MiR398 was downregulated under biotic and abiotic stresses in tomato (Luan and Liu, 2014). The main goal of our study was to investigate whether sRNA production is affected by the *7B-1* mutation, and if miRNAs are involved in the regulation of abiotic stress response in *7B-1*. Known and novel miRNAs and their targets were identified and their expressions were studied in stress-treated *7B-1* and WT seedlings. Targets of miRNAs with potential roles in regulation of stress response were validated using 5′-RACE.

## Materials and Methods

### Plant Materials and Stress Treatments

The *7B-1* mutant and WT seedlings were grown under continuous BL in temperature-controlled growth chamber set at 23°C (Microclima 1000E, Snijders Scientific B.V). Abscisic acid, mannitol, and salt treatments were performed as described by Fellner and Sawhney, (2002) and Li et al. (2011). Two-week-old seedlings grown on Murashige and Skoog medium under BL were transferred into mediums supplemented simultaneously with 10 μM ABA and 140 mM mannitol, incubated for 24 h, and subsequently used for construction of sRNA libraries. For quantitative polymerase chain reaction (qPCR) analysis, 2-wk BL-grown seedlings were transferred to mediums containing 10 μM ABA, 140 mM mannitol, or 120 mM NaCl and incubated for 0, 2, 4, 8, 12, and 24 h under BL. Cold treatment was performed as described by Cao et al. (2014) by incubating the seedlings at 4°C for 0 to 24 h under BL. Wild-type seedlings were subjected to similar stress conditions and served as control.

## Small RNA Libraries

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) from stress-treated *7B-1* and WT seedlings after 24 h and pooled separately in equimolar ratio. Two sRNA libraries were constructed using the TruSeq Small RNA Sample Preparation Kit (Illumina). In brief, sRNA fractions with size of 18 to 30 nt were isolated from 15% denaturing polyacrylamide gels, ligated to the 5′ and 3′ TruSeq adaptors and then converted to DNA by reverse transcription–polymerase chain reaction (RT-PCR) following the kit protocol. The final PCR products were gel purified and sequenced using Illumina Hiseq2000 platform (Illumina).

## Sequence Analysis

Adaptor sequences were removed and reads were mapped (no mismatch allowed) to the tomato genome (ITAG2.4 Release) using PatMaN (Prufer et al., 2008) and custom-made Perl scripts. Sequences that matched ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs) in Rfam and National Center for Biotechnology Information (NCBI) nt/nr databases were identified. Known miRNAs were identified using miRprof software (Moxon et al., 2008b) available from the UEA sRNA workbench (http://srna-tools.cmp.uea.ac.uk/) allowing up to two mismatches with the mature miRNA sequences in the miRBase database release 19 (Kozomara and Griffiths-Jones, 2011). Novel miRNAs were predicted using miRCat software (UEA sRNA workbench), and their secondary structures were analyzed using a RNA hairpin folding tool (UEA sRNA workbench). Only miRNAs with perfect hairpin structures that met the criteria for miRNAs were regarded as novel miRNAs candidates. MicroRNA targets were predicted using the tomato ITAG cDNA v2.3, allowing up to four mismatches, and no mismatch at the 10 to 11 nt from the 5′ end of the miRNA. The two libraries were normalized using the reads-per-million approach (Mortazavi et al., 2008), and differential expression values were calculated as $\log_2$ of offset-fold changes as described by Mohorianu et al. (2011). Phased secondary small interfering RNAs were identified based on their phased expression to genomic loci of interest by mapping the sRNA reads to the tomato ITAG cDNA v2.3 using integrative genomics viewer (IGV) software (https://www.broadinstitute.org/igv/). Gene ontologies of miRNA targets were assigned based on biological functions using the Blast2 go tool (http://www.blast2go.com/b2 ghome). The sequences could be found under accession numbers GSE65964 and GSE65788.

## Quantitative Polymerase Chain Reaction

Expressions of miRNAs were validated in BL-grown *7B-1* hypocotyls and roots in response to ABA, mannitol, NaCl and cold treatments using the MiR-X miRNA First-Strand Synthesis and SYBR qRT-PCR kit (Clontech). In a single reaction, sRNAs were polyadenylated and reverse transcribed using poly(A) polymerase and SMART MMLV Reverse Transcriptase provided by the kit. MicroRNA-specific forward primers are listed in Supplemental Table S4. The U6 snRNA was used in data normalization as a reference. Quantitative PCRs were performed at 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, and annealing and extension at 60°C for 20 s. Changes of expressions were calculated as normalized fold ratios using the ΔΔCT method (Livak and Schmittgen, 2001). Quantitative PCR analysis of miRNA targets were performed using the SensiFAST SYBR Lo-ROX kit (Bioline). First-strand cDNAs were synthesized using the PrimeScript First Strand cDNA Synthesis kit (TAKARA). Gene-specific primers were designed flanking the miRNA cleavage sites (Supplemental Table S4). Tomato α-*tubulin* and *CAC* housekeeping genes were used as references for data normalization (data were shown only for α-*tubulin*). The PCR conditions were set at 95°C for 2 min, followed by 40 cycles of 95°C for 5 s, and annealing and extension at 60°C for 20 s.

**Table 1. Statistics of small RNA (sRNA) reads.**

| | Wild-type stress | | *7B-1* stress | |
|---|---|---|---|---|
| sRNA reads[†] | Total | Unique | Total | Unique |
| Raw reads | 63,407,599 | | 54,879,187 | |
| Quality-filtered | 63,303,274 | | 54,769,783 | |
| Adaptor-removed | 27,846,319 | | 43,891,268 | |
| Genome-matched | 22,027,376 | 548,298 | 7099,045 | 273,292 |
| rRNA | 1167,034 (5.3%) | 31,129 (5.68%) | 478,007 (6.73%) | 23,689 (8.67%) |
| tRNA | 686,021 (3.1%) | 3658 (0.67%) | 162,263 (2.29%) | 2664 (0.97%) |
| snoRNA | 30,713 (0.14%) | 2036 (0.37%) | 6898 (0.1%) | 1300 (0.48%) |
| snRNA | 24,320 (0.11%) | 700 (0.13%) | 7080 (0.1%) | 490 (0.18%) |

[†] rRNA, ribosomal RNA; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; tRNA, transfer RNA.

## Rapid Amplification of 5′ Complementary DNA Ends Analysis

MicroRNA targets were validated by amplification of 5′ cDNA ends using the GeneRacer kit (Invitrogen). In brief, mRNA was purified from 5 μg of total RNA, ligated to the 5′ RNA adaptor having a 5′ free phosphoric acid, and then reverse transcribed. Subsequently, 1 μL of 10× diluted reverse transcription product was used to amplify the 5′ end of the corresponding targets using the 5′ GeneRacer and 3′ gene-specific primers (Supplemental Table S5). Amplified products were analyzed on an agarose gel, purified, and cloned into the pCR4-TOPO vector (Invitrogen). Ten different colonies were subjected to sequence analysis.

# Results

## Sequencing of Small RNAs

Two sRNA libraries from BL-grown WT and *7B-1* seedlings treated simultaneously with ABA and mannitol were constructed and sequenced, which produced about 63 and 54 million raw reads, respectively. After quality check and adaptor trimming, reads were mapped to the tomato ('Heinz') genome and an overview of the read numbers is shown in Table 1. The majority of reads were 21 to 24 nt, with the 24-nt class being the most abundant group of nonredundant sRNAs (Fig. 1). Higher

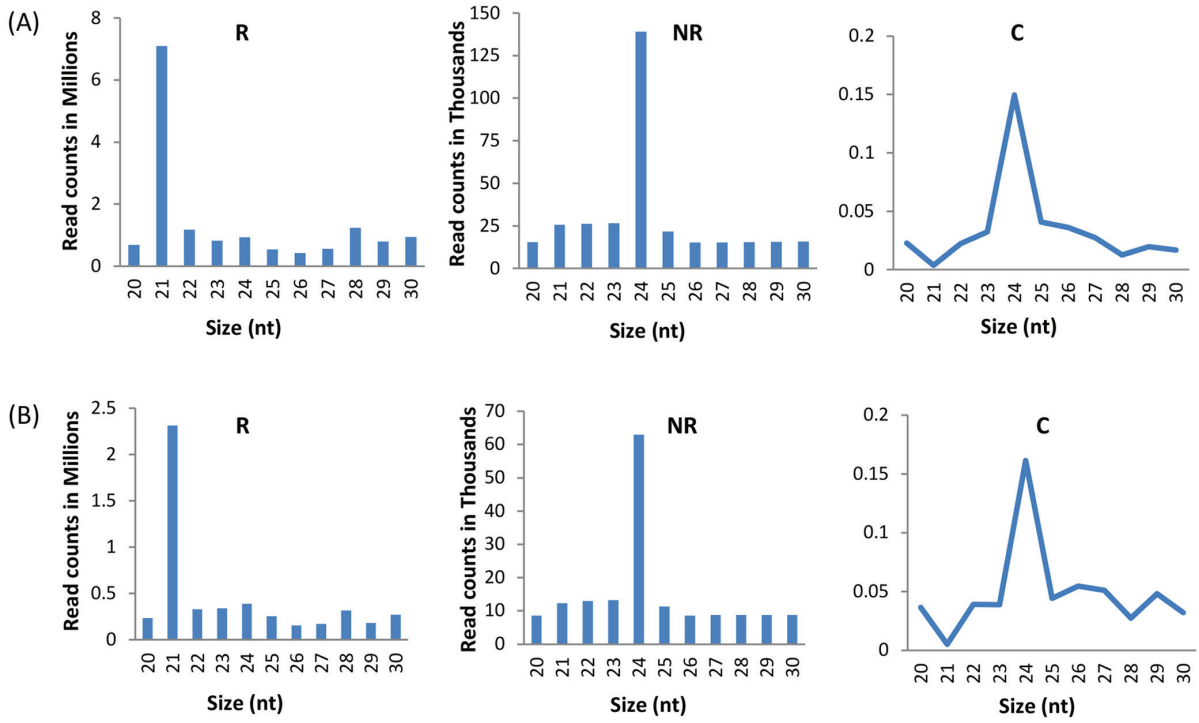


Figure 1. Size distribution of redundant (*R*) and nonredundant (*NR*) small RNA reads and their complexities (*C*) in wild-type (panel A) and *7B-1* (panel B) stress-treated seedlings.

abundance of the 24-nt class is consistent with previous reports from other species (Mohorianu et al., 2011; Gao et al., 2012; Wei et al., 2013; Zhang et al., 2013; Aryal et al., 2014), except for grapevine (*Vitis vinifera* L.) (Pantaleo et al., 2010) and *Brassica juncea* (L.) Czern. (Yang et al., 2013). Population complexity of sRNA libraries was defined as the ratio of unique/total reads, where the low complexity of the 21-nt class in our libraries implied that a relatively small number of unique reads were highly expressed in contrast to the 24-nt class (Fig. 1). This is consistent with the biogenesis of 24-nt heterochromatin-associated sRNAs and their cloud-shape distribution over the loci (Schwach et al., 2009).

## MicroRNA Analysis

Known miRNAs were identified using the miRBase database release 19 (Kozomara and Griffiths-Jones, 2011). A total of 305 miRNA variants, representing 29 families of known miRNAs, and 303 miRNA, variants representing 27 families, were identified from WT and *7B-1* stress-treated seedlings, respectively (Supplemental Table S1). MiR2118 and miR2218 were absent from *7B-1* library, however they were detected in *7B-1* seedlings using qRT-PCR. Except miR2118 and miR2218 families, the rest of the families had multiple members and were present in both libraries (Supplemental Table S1). MiR166 with 219 members comprised the biggest family, accounting for 97.9 and 98.9% of the total miRNA-matching reads in WT and *7B-1* libraries, respectively. A total of 27 novel miRNAs were identified from the two libraries, including 13 from WT, 8 from *7B-1*, and 6 in common from both libraries (Supplemental Table S2, S3). MicroRNA with star sequences were identified for two novel miRNAs. Analysis of miRNA precursors showed that they could make near-perfect hairpin structures (Supplemental Fig. S1, S2).

To identify miRNAs linked to the BL-specific higher tolerance of *7B-1* to abiotic stress, expressions of miRNAs were compared between stress-treated *7B-1* and WT seedlings grown under BL. MiR5300, miR5301, and miR2916 were upregulated and miR159, miR166, miR472, and miR482 were downregulated in stress-treated *7B-1* seedlings (Table 2). Figure 2 shows the number of miRNAs in each miRNA family containing at least one miRNA differentially expressed between *7B-1* and WT libraries. Out of the total of 27 novel miRNAs, three (denoted miR#A, miR#C, and miR#D) were differentially expressed. MiR#A and miR#D were down- and miR#C was upregulated, respectively, in stress-treated *7B-1* seedlings (Supplemental Table S2, S3). Using qRT-PCR, expressions of miR159, miR166, miR472, miR482, miR#A, and miR#D were further analyzed in response to ABA, mannitol, NaCl, and cold after 24 h of treatments (Fig. 3, 4). MiR159, miR166, miR472, and miR482 were not differentially expressed between *7B-1* and WT hypocotyls and roots under control condition. MiR159 was downregulated in *7B-1* hypocotyl and more strongly in root in response to all treatments. MiR166 was downregulated in *7B-1* hypocotyl and root by ABA, mannitol, and NaCl, but upregulated by cold treatment. MiR472 and miR482 were downregulated in *7B-1* hypocotyl and more strongly in root in response to ABA, mannitol, and NaCl, but not to cold treatment. In the control condition, miR#A had a lower expression in *7B-1* hypocotyl and root than the WT, while miR#C and miR#D were not

**Table 2. List of differentially expressed microRNAs (miRNAs) between wild-type and *7B-1* stress-treated seedlings.**

| | | Read counts | | Normalized reads | | DE† |
|---|---|---|---|---|---|---|
| miRNA | Sequence | WT_stress | *7B-1*_stress | WT_stress | *7B-1*_stress | WT_stress/*7B-1*_stress |
| miR5300 | CCCCAGTCCAGGCATTCCAAC | 133 | 172 | 60.4 | 242.3 | –1.7 |
| miR2916 | GGGGGCTCGAAGACGATCAGAT | 25 | 41 | 11.3 | 57.8 | –1.3 |
| miR5301 | TGTGGGTGGGGTGGAAAGATT | 29 | 33 | 13.2 | 46.5 | –1.0 |
| miR166 | TCGGACCAGGCTTCATTCCTC | 137,102 | 20,408 | 62,241.6 | 28,747.5 | 1.1 |
| miR159 | TTTGGATTGAAGGGAGCTCTA | 15,775 | 2,532 | 7,161.5 | 3,566.7 | 1.2 |
| miR482 | TCTTGCCAATACCGCCCATTCC | 3,844 | 433 | 1,745.1 | 609.9 | 1.5 |
| miR472 | TCTTTCCTACTCCGCCCATACC | 84,859 | 8,135 | 38,524.3 | 11,459.3 | 1.7 |

† DE, differential expression values, calculated as log2 offset fold changes (with an offset of 20) on the normalized expression levels. Negative and positive values mean up- and downregulation of the expression in stress-treated *7B-1* seedlings, respectively. DE value of ± 1 was considered as a cutoff value for significant changes of the expression.

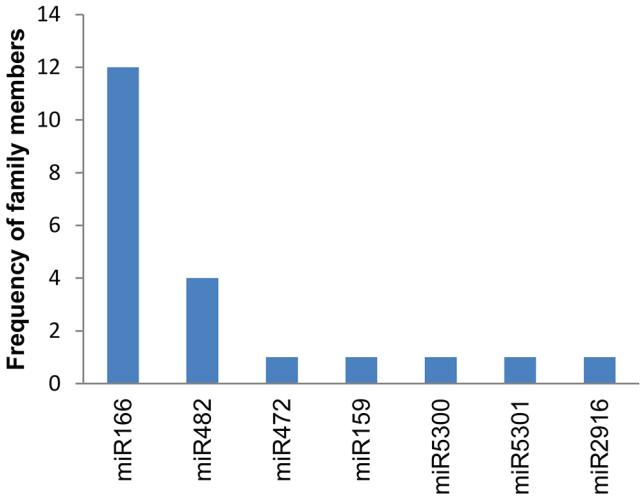


Figure 2. Number of differentially regulated microRNA members in each family.

differentially expressed (Fig. 4). MiR#A and miR#D were both downregulated in *7B-1* hypocotyl and root by ABA, mannitol, NaCl, but not by cold treatment. MiR#C was upregulated only in *7B-1* root in response to ABA, mannitol, and NaCl but not to cold treatment. This suggests that miR#C may regulate different biological process in hypocotyl and root tissues.

## Analysis of MicroRNA Targets

Putative targets of differentially expressed miRNAs were identified (Table 3, 4). In addition to the validated miRNA targets from the literature, a number of new putative targets were also computationally identified, and those of interest were further validated using 5′-RACE. Based on the biological processes the genes were involved in, miRNA targets were categorized into 13 classes (Fig. 5) with cellular process, metabolic process and response to stimulus comprising the biggest classes. Among the targets of known miRNAs (Table 3) were encoded stress-related proteins, including serine–threonine protein kinase, BHLH transcription factor, GDSL esterase, HD-Zip III, adenosine triphosphate-binding cassette (ABC) transporter, GAMYBL1/2, WD-40, G proteins, and defense-related NBS-LRR proteins (McHale et al., 2006; Afzal et al., 2008; Dai et al., 2008; Zhou et al., 2009; Jiang et al., 2012; Colaneri et al., 2014; Kong et al., 2015; Yang et al., 2014). Among the targets of novel miRNAs (Table 4) were stress-related proteins, including receptor-like kinase, PHD finger, and ABC transporter proteins (Afzal et al., 2008; Wei et al., 2009; Nguyen et al., 2014). Putative targets of miR159, miR166, miR472, miR482, miR#A, and miR#D were validated in *7B-1* hypocotyl and root by 5′-RACE. Sequence analysis showed (Fig. 6) that the 5′ ends of the cleaved *GAMYBL1*, *HD-Zip III*, *TIR-NBS-LRR*, and *CC-NBS-LRR* transcripts corresponded to nucleotide complementary to the 10th nucleotide of miR159, miR166, miR472, and miR482, respectively.

MiR#A cleaved *receptor-like kinase* transcripts. MiR#D cleaved *ABC transporter* transcripts, but not *PHD finger* transcripts. The results confirmed that these miRNAs regulate their targets through the cleavage of their transcripts in *7B-1* hypocotyl and root.

Figure 7 shows qRT-PCR analysis of known miRNA targets in BL-grown *7B-1* hypocotyl and root in response to ABA, mannitol, NaCl, and cold after 24 h of treatments. In control condition (no stress), miRNA targets were not differentially regulated between *7B-1* and WT hypocotyls and roots. In stress condition, *GAMYBL1* (miR159 target) was upregulated in hypocotyl and root by ABA, mannitol, NaCl, and slightly by cold treatment. *HD-Zip III* (miR166 target) was upregulated by ABA, mannitol, and NaCl more strongly in hypocotyl than root, while it was downregulated by cold in hypocotyl and root. *TIR-NBS-LRR* (miR472 target) and *CC-NBS-LRR* (miR482 target) were both upregulated in hypocotyl and root in response to ABA, mannitol, NaCl, but not cold treatment. Primary transcripts of *receptor-like kinase* (miR#A target) were found more abundantly in *7B-1* hypocotyl and root than the WT in control condition (Fig. 8), while *ABC transporter* (miR#D target) was not differentially expressed. In stress condition, *receptor-like kinase* was upregulated in hypocotyl and root by ABA, mannitol, and NaCl, but not cold treatment. *ABC transporter* was upregulated in hypocotyl and root more strongly by ABA, mannitol, and NaCl and slightly by cold treatment. Differential regulation of these stress-related genes could be associated with higher tolerance of *7B-1* to abiotic stress in BL than the WT.

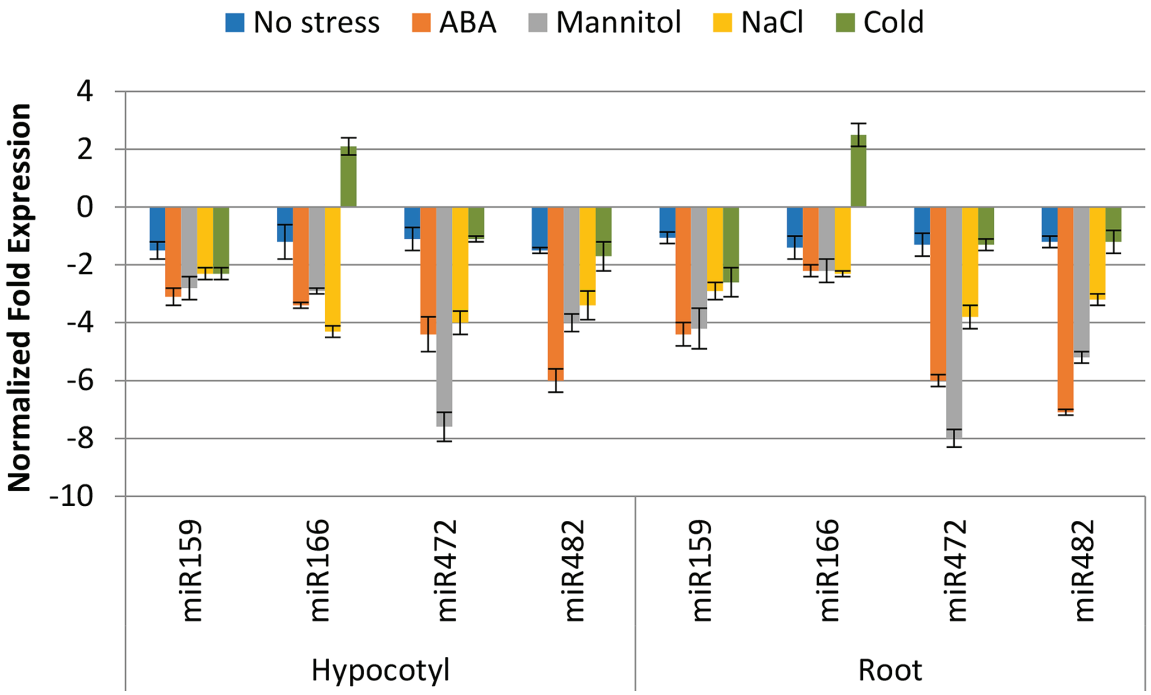


Figure 3. Quantitative reverse transcription–polymerase chain reaction analysis of differentially expressed known microRNAs. Expression changes are presented as normalized fold changes between *7B-1* and wild-type (reference tissue) in no stress and stress conditions (after 24 h of treatments). Positive and negative values indicate up- and downregulation of the expression, respectively. Two-fold threshold was considered as a cutoff value for significant changes in the expression. Error bars represent standard errors of three technical replicates based on Duncan's new multiple range test at $p = 0.05$.

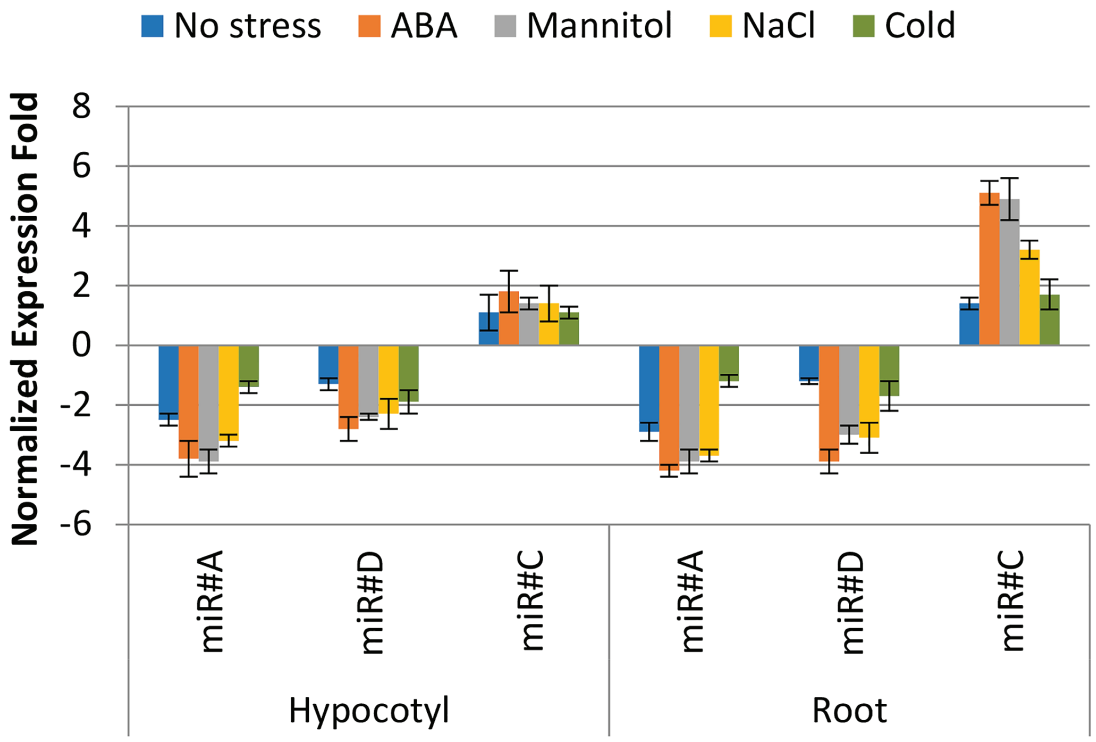


Figure 4. Quantitative reverse transcription–polymerase chain reaction analysis of differentially expressed novel microRNAs. Expression changes are presented as normalized fold changes between *7B-1* and wild-type (reference tissue) in no stress and stress conditions (after 24 h of treatments). Positive and negative values indicate up- and downregulation of the expression, respectively. Two-fold threshold was considered as a cutoff value for significant changes in the expression. Error bars represent standard errors of three technical replicates based on Duncan's new multiple range test at $p = 0.05$.

**Table 3. List of the predicted targets of differentially expressed known microRNAs (miRNAs).**

| miRNA | Target gene accession | Annotation |
|---|---|---|
| miR5300 | Solyc02g081470.2.1 | Serine–threonine protein kinase |
| | Solyc02g070780.2.1 | DNA replication licensing factor MCM3 |
| | Solyc03g115540.1.1 | BHLH transcription factor |
| | Solyc03g005660.2.1 | CC-NBS-LRR† |
| | Solyc04g080830.2.1 | Pentatricopeptide repeat-containing protein† |
| | Solyc11g007770.1.1 | Exostosin-2 |
| | Solyc11g012970.1.1 | Aminoacylase-1 |
| miR5301 | Solyc03g116460.2.1 | Aspartate racemase |
| | Solyc04g011350.2.1 | 2-oxoglutarate dehydrogenase E1 component |
| | Solyc05g043320.1.1 | GDSL esterase |
| | Solyc08g075510.2.1 | Transcription factor jumonji |
| | Solyc09g083200.2.1 | Nod factor receptor protein |
| | Solyc09g083210.2.1 | Receptor-like protein kinase |
| miR166 | Solyc02g024070.2.1 | HD-Zip III† |
| | Solyc03g006970.1.1 | Subtilisin |
| | Solyc03g044180.1.1 | ABC transporter |
| | Solyc07g045410.1.1 | Pentatricopeptide repeat-containing protein |
| | Solyc08g066410.1.1 | Serine/threonine protein kinase |
| | Solyc10g083110.1.1 | Dihydrodipicolinate synthase 2 |
| miR159 | Solyc01g009070.2.1 | GAMYBL1 |
| | Solyc01g102510.2.1 | WD-40 |
| | Solyc02g078670.2.1 | COP1-Interacting Protein 7 |
| | Solyc02g090160.2.1 | G protein |
| | Solyc03g043890.2.1 | Aminotransferases |
| | Solyc06g073640.2.1 | GAMYBL2 |
| | Solyc07g052640.2.1 | Glycosyltransferase-like protein |
| | Solyc09g082890.1.1 | Calcium-transporting ATPase 1 |
| | Solyc10g019260.1.1 | MYB39-like |
| miR482 | Solyc05g008070.2.1 | CC-NBS-LRR† |
| | Solyc07g037950.1.1 | Phosphodiesterase |
| | Solyc08g079730.1.1 | Mate efflux family protein |
| | Solyc09g091990.2.1 | Serine/threonine protein kinase |
| | Solyc10g084590.2.1 | Cytochrome P450 |
| | Solyc11g008140.1.1 | Pectate lyase family protein |
| | Solyc12g056490.1.1 | WD40 repeat |
| | Solyc12g056960.1.1 | Glycoside hydrolase |
| | Solyc03g112630.2.1 | Fas-associated factor 1-like protein |
| miR472 | Solyc05g006630.2.1 | TIR-NBS-LRR† |
| | Solyc04g015210.2.1 | CC-NBS-LRR† |
| | Solyc04g025160.2.1 | ATPase |
| | Solyc08g005410.2.1 | Kinase-START 1 |
| | Solyc10g007200.2.1 | Beta-1 3-galactosyltransferase 6 |
| | Solyc11g011560.1.1 | Zinc finger |
| | Solyc01g097390.2.1 | Aldo/keto reductase |

† Indicates targets that have identified from multiple loci.

**Table 4. List of the predicted targets of novel microRNAs (miRNAs).**

| miRNA | Target gene accession | Annotation |
|---|---|---|
| miR#A | Solyc11g008040.1.1 | Pullulanase |
| | Solyc03g115420.1.1 | F-box domain containing protein |
| | Solyc06g075030.1.1 | Receptor-like kinase |
| miR#B | Solyc08g006010.2.1 | WD40 repeat-like |
| | Solyc08g014040.2.1 | Methyltransferase-16 |
| | Solyc10g007210.1.1 | LRR receptor-like kinase |
| | Solyc11g008040.1.1 | Pullulanase |
| | Solyc01g100790.1.1 | Pentatricopeptide repeat |
| miR#C | Solyc03g025280.2.1 | RNA-binding protein |
| miR#D | Solyc05g005640.2.1 | PHD finger family protein |
| | Solyc06g036490.1.1 | ABC transporter |

## MicroRNA–Target Correlation in Response to Stress

Expressions of miRNAs and their targets as well as their correlations were further studied in the hypocotyls of *7B-1* and WT seedlings grown under BL in response to ABA and mannitol at 0, 0.5, 2, 4, 8, 12, and 24 h after treatments (Fig. 9, 10). A consistent negative correlation was observed between miRNA levels and expression of their targets in response to both treatments over the time series. Expressions of miR159, miR166, miR472, and miR482 decreased noticeably (Fig. 9) only after 2 h of treatments, reached to their lowest points at 8 to 12 h, and then remained constant till 24 h. Targets of these miRNAs were upregulated after 2 h of treatments, reaching their highest points at 8 to 12 h, where the expressions remained constant till 24 h. As mentioned earlier, miR#A had a lower expression level in untreated *7B-1* seedlings than WT. MiR#A expression further decreased (Fig. 10) as early as 0.5 h after each treatments, dropped into its lowest point at 4 h after ABA, and 8 h after mannitol treatments. It slightly increased till 8 h after ABA and 12 h after mannitol treatments, where remained almost constant till 24 h. MiR#D expression decreased after 2 h, reached to its lowest points at 4 h, and then remained constant till 24 h. *Kinase* (miR#A target) expression increased drastically as early as 0.5 h of each treatments, reached to its highest point at 8 h, and decreased after 12 h. *ABC transporter* (miR#D target) expression increased after 2 h of each treatments, peaked at 8 to 12 h, and then remained almost constant. These observations suggest that while miR156, miR166, miR472, miR482, and miR#D might regulate *7B-1* response after 2 h of stress onset, miR#A and its target are tightly connected to the early stress response in *7B-1* caused by ABA and mannitol.

## Discussion

To investigate the role of miRNAs in regulation of stress response in *7B-1*, two sRNA libraries from *7B-1* and WT seedlings, which had been treated simultaneously with ABA and mannitol, were sequenced and expression

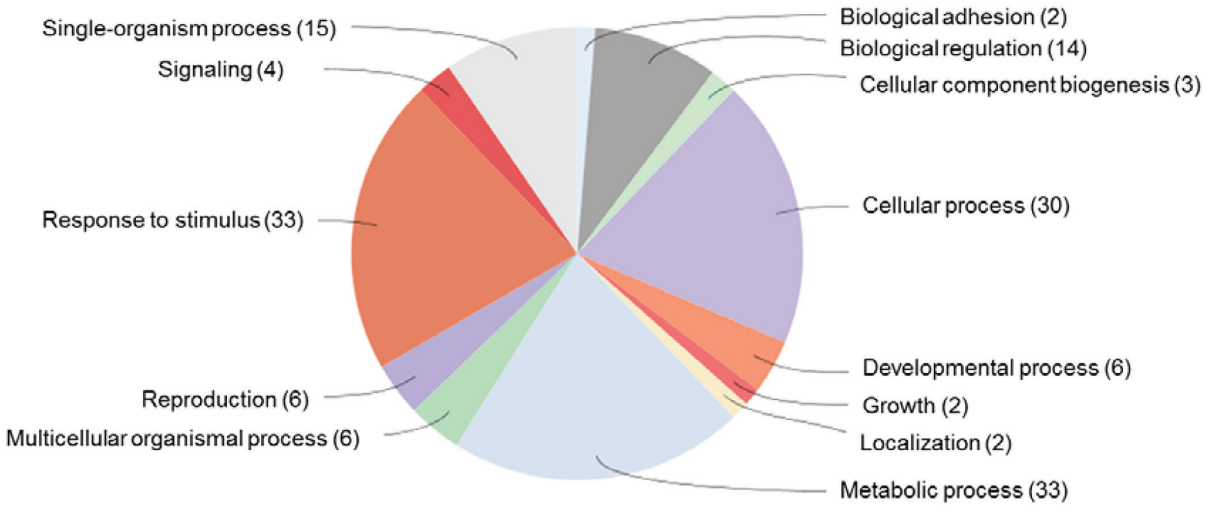


Figure 5. Gene ontology of microRNA targets. Predicted targets were categorized into different biological classes and numbers in the parenthesis indicate the frequency of members in each category.

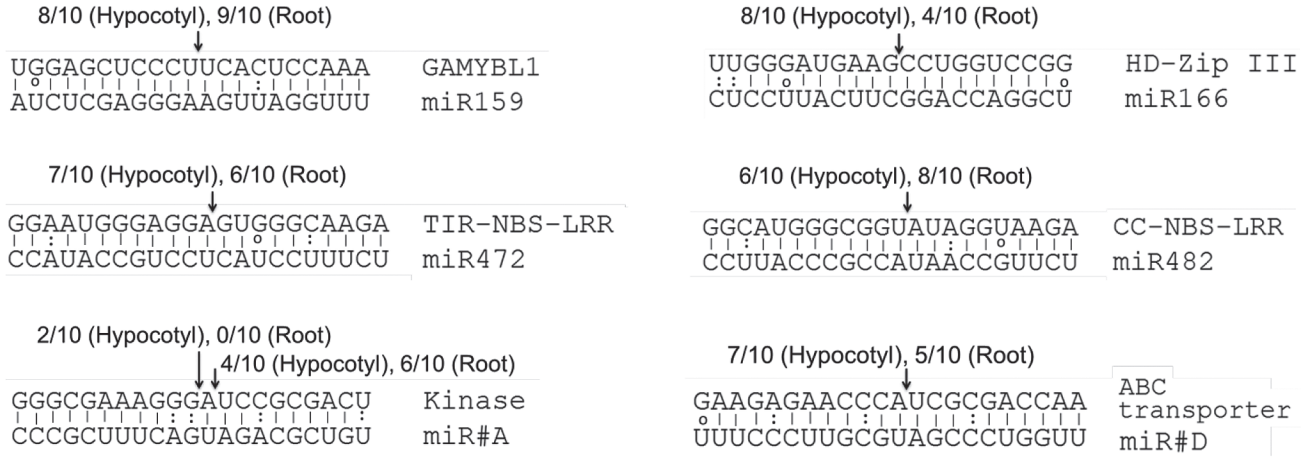


Figure 6. Rapid amplification of 5′ complementary DNA ends (5′-RACE) validation of microRNA (miRNA) targets in *7B-1* hypocotyl and root. Gene transcripts are in 5′-3′ and miRNAs in 3′-5′ directions. The arrows indicate the cleavage sites of target messenger RNA and numbers above them indicate frequency (out of 10) of sequences found at the exact miRNAs cleavage sites. Watson–Crick pairing (vertical dashes), guanine–uracil wobble pairing (circles), and other mismatches (:) are indicated.

profiles of miRNAs were compared. Size distribution and complexity of sRNAs were similar in the two libraries, where the 24 nt class was the most abundant group of sRNAs, followed by the 23, 22, and 21 nt classes. Twenty-nine families of known miRNA were identified. MiR5300, miR5301, and miR2916 were upregulated and miR159, miR166, miR472, and miR482 were downregulated in *7B-1* library. Out of the total of 27 identified novel miRNAs, only three were differentially expressed (miR#A, miR#C, and miR#D). MiR5300 and miR5301 were recently identified from tomato fruit (Mohorianu et al., 2011), but their function is still uncharacterized. We did not find any target for miR2916 using target prediction programs, although the same approach identified potential targets for all other miRNAs we studied. Other studies found many different sRNAs mapping to the putative pre-miR2916 sequence, suggesting the sequence could be a siRNA and not miRNA (Huang et al., 2013), therefore we did not investigate this miRNA further. MiR159, miR166, miR472, miR482, miR#A, and miR#D were those of particular interest in our study, as their targets had potential roles in plant response and adaptation to abiotic stress. Targets of these miRNAs were validated by 5′-RACE and expressions of these miRNAs and their targets were further investigated in hypocotyls and roots of *7B-1* and WT seedlings grown under BL in response to ABA, mannitol, NaCl, and cold treatments.

MiR159, miR166, miR472, and miR482 were differentially expressed between *7B-1* and WT seedlings in both hypocotyl and root tissues in response to ABA, mannitol, and NaCl, while cold treatment only affected miR159 and miR166. These miRNAs were not differentially expressed between WT and *7B-1* hypocotyls and roots in the absence of stress. These observations suggest

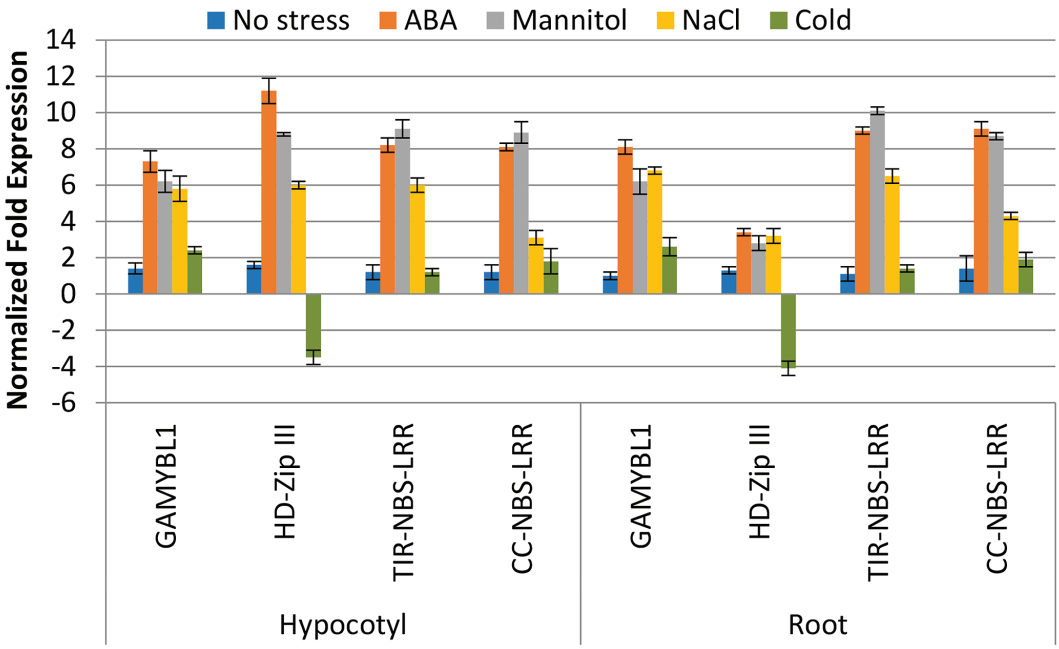


Figure 7. Quantitative reverse transcription–polymerase chain reaction analysis of known microRNA targets. Expression changes are presented as normalized fold changes between *7B-1* and wild-type (reference tissue) in no stress and stress conditions (after 24 h of treatments). Positive and negative values indicate up- and downregulation of the expression, respectively. Two-fold threshold was considered as a cutoff value for significant changes in the expression. Error bars represent standard errors of three technical replicates based on Duncan's new multiple range test at $p = 0.05$.

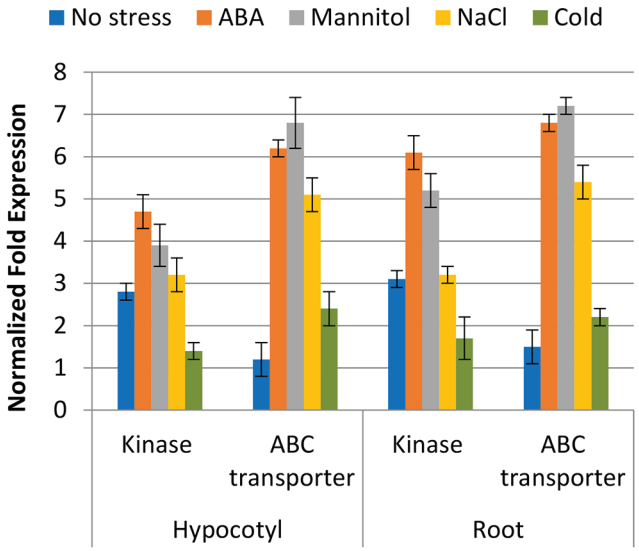


Figure 8. Quantitative reverse transcription–polymerase chain reaction analysis of novel microRNA targets. Expression changes are presented as normalized fold changes between *7B-1* and wild-type (reference tissue) in no stress and stress conditions (after 24 h of treatments). Positive and negative values indicate up- and downregulation of the expression, respectively. Two-fold threshold was considered as a cutoff value for significant changes in the expression. Error bars represent standard errors of three technical replicates based on Duncan's new multiple range test at $p = 0.05$.

that these miRNAs might regulate the *7B-1* response to ABA, mannitol, NaCl, and cold and facilitate the enhanced stress tolerance of *7B-1* under BL compared with the WT. MiR#A and miR#D were also downregulated in *7B-1* hypocotyl and root by all treatments, except cold. Interestingly, miR#C expression remained unchanged in hypocotyl but strongly induced in root by ABA, mannitol, and NaCl. This suggests a stress-related role for this miRNA in root but not hypocotyl.

MiR159 targets several *MYBs* including *MYB33*, *MYB65*, *MYB101*, and *GAMYBL1/2* (Millar and Gubler, 2005; Palatnik et al., 2003). Overexpression of miR159 suppressed *MYB33* and *MYB101* transcript levels in *Arabidopsis* and renders plants hypersensitive to ABA (Reyes and Chua, 2007). Rapid amplification of 5′ complementary DNA ends analysis in our study showed that miR159 directed the cleavage of *GAMYBL1* transcripts out of the four predicted *MYB* targets in hypocotyl and root. *GAMYBL1* and *2* play important roles in seed development in tomato, rice and *Arabidopsis* (Kaneko et al., 2004; Reyes and Chua, 2007; Gong and Bewley, 2008), but none were functionally characterized with respect to stress response. MiR159 was downregulated in *7B-1* by ABA, mannitol, NaCl, and cold treatments and expression of *GAMYBL1* was negatively correlated with the miR159 level, but understanding how upregulation of *GAMYBL1* is connected to stress tolerance in *7B-1* under BL requires further functional studies.

Cleavage of *HD-Zip III* transcripts by miR166 was confirmed by 5′-RACE in *7B-1* hypocotyl and root. Some studies have reported upregulation of miR166 by abiotic stresses such as drought, salinity, and cold (Trindade et al., 2010; Kong et al., 2010; Cao et al., 2014), while others described downregulation of miR166 by such stresses

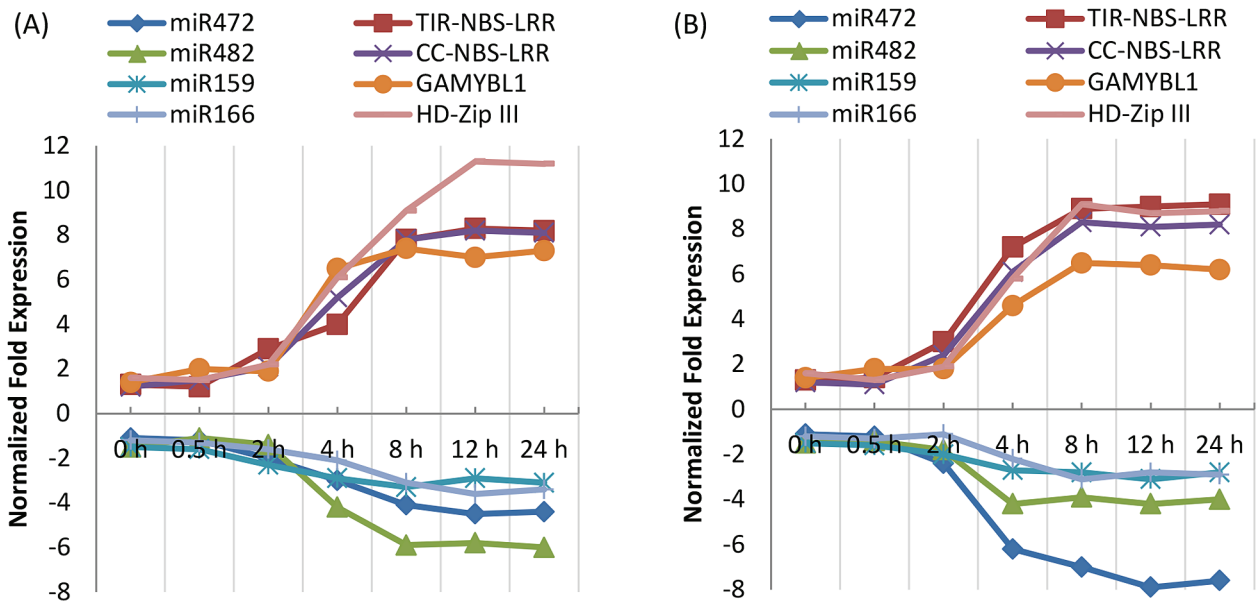


Figure 9. Correlation of miR159, miR166, miR472, miR482 and their targets in response to ABA (panel A) and mannitol (panel B). Expression changes are presented as normalized fold changes between *7B-1* and wild-type (reference tissue) in no stress and stress conditions. Positive and negative values indicate up- and downregulation of the expression, respectively. Two-fold threshold was considered as a cutoff value for significant changes in the expression.

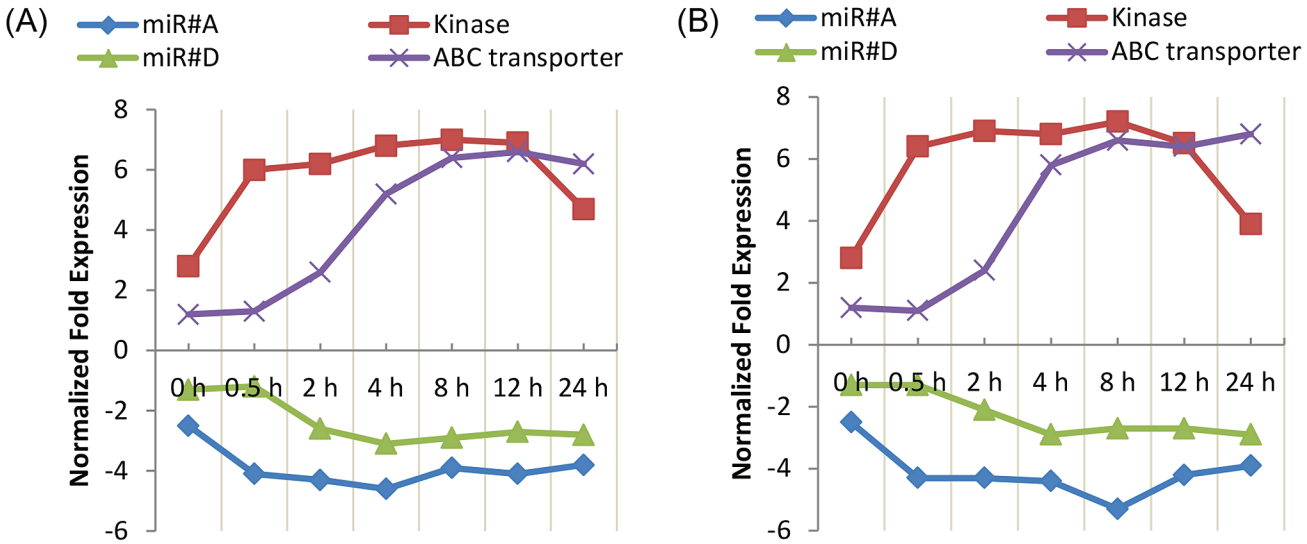


Figure 10. Correlation of miR#A, miR#D and their targets in response to ABA (panel A) and mannitol (panel B). Expression changes are presented as normalized fold changes between *7B-1* and wild-type (reference tissue) in no stress and stress conditions. Positive and negative values indicate up- and downregulation of the expression, respectively. Two-fold threshold was considered as a cutoff value for significant changes in the expression.

(Ding et al., 2009; Kantar et al., 2010). HD-Zip III family of transcription factors are generally considered as nonstress-related proteins, which regulate axillary meristem initiation and leaf development (Boualem et al., 2008). Although few reports have documented differential expression of *HD-Zip III* genes in response to abiotic stresses, such as drought and salinity (Belamkar et al., 2014; Chen et al., 2014), none have characterized the function of these genes in respect to stress response in plants. Upregulation of *HD-Zip III* under BL in response to ABA, mannitol, and NaCl and downregulation by cold is likely associated with stress-tolerant phenotype of *7B-1*, but unraveling the actual function of this gene requires further analysis.

Several studies have reported upregulation of miR472 and miR482 by abiotic stresses, such as drought and salt stresses (Lu et al., 2008; Shuai et al., 2013), while others indicated downregulation of these miRNAs by cold, heat, and drought stresses (Li et al., 2011). MiR472 and miR482 directed the cleavage of *TIR-NBS-LRR* and *CC-NBS-LRR*

transcripts in *7B-1* hypocotyl and root. NBS-LRRs are disease resistance proteins, which regulate defense-related responses in plants (Karlova et al., 2013; Shuai et al., 2013). Although differential expression of miR472 and miR482 by abiotic stresses suggests a stress-related role for these miRNAs, data on functional characterization of miR472- and miR482-NBS-LRRs cleavage cascades with respect to abiotic stress response in plants is scarce. Wan et al. (2012) identified several *NBS-LRRs* in pepper (*Capsicum annuum* L.), which were upregulated by ABA. Several *NBS-LRRs* in *Arabidopsis* were upregulated under heat and drought stresses (Prasch and Sonnewald, 2013). Overexpression of a *NBS-LRR* gene in *Arabidopsis* conferred significant drought tolerance (Chini et al., 2004). MiR472- and miR482-guided upregulation of *TIR-NBS-LRR* and *CC-NBS-LRR* under BL by ABA, mannitol, and NaCl is likely to contribute to higher stress tolerance in *7B-1*; nevertheless, functional characterization of these genes is required to understand their functions.

MiR472/482-mediated cleavage of *NBS-LRRs* not only plays an important role in regulation of disease resistance but also triggers production of phasiRNAs that are able to regulate the expression of their targets as well as other *NBS-LRRs* in *trans* (Zhai et al., 2011; Shivaprasad et al., 2012). To identify *NBS-LRRs*-derived phasiRNAs triggered by miR472/482-cleavage, we analyzed sRNAs with phased expression to *NBS-LRR* loci; however, we did not find any *NBS-LRR*-mapping phasiRNAs. This indicates that production of phasiRNAs from *NBS-LRR* loci is not an active mechanism in *7B-1* response to abiotic stress.

While targets of conserved miRNAs seem to be conserved across species, intriguingly, some miRNA family members seem to behave in opposite ways in different tissues or species in response to similar stimuli. It is possible that differences in plant developmental stage, stress condition, and plant sensitivity to stress could contribute to differential regulation of miRNAs. It is also possible that factors involved in RNA metabolism could affect processes indirectly related to miRNA action and biogenesis (Kim et al., 2008; Rogers and Chen, 2013). Our knowledge of miRNA families in plants is not yet saturated, as novel miRNAs are still being continually identified from different species, including tomato (Pilcher et al., 2007; Moxon et al., 2008a; Mohorianu et al., 2011). We identified a number of potential novel miRNAs and their presence was confirmed in our libraries using qRT-PCR. They all formed near-perfect hairpin structures. MiR#A and miR#D directed the cleavage of *receptor-like kinase* and *ABC transporter* transcript, respectively. *Kinases* and *ABC transporters* are well documented in regulation of plant response to a wide range of abiotic stresses (Osakabe et al., 2013; Zhang et al., 2013; Nguyen et al., 2014). Upregulation of these genes could enhance the *7B-1* tolerance to abiotic stress.

A consistent negative correlation was observed between expression levels of miRNAs and their targets in BL-grown *7B-1* hypocotyl in response to ABA and mannitol over a time series. Levels of miR159, miR166, miR472, miR482, and miR#D decreased after 2 h of each treatment, and their targets were upregulated accordingly at about the same time. In contrast, miR#A-*kinase* response to ABA and mannitol was much earlier (0.5 h after treatments), which suggests that miR#A mediates the early stress response in *7B-1*. Overall, we identified known and novel miRNAs, which are associated and likely to facilitate the enhanced tolerance of *7B-1* to abiotic stress under BL. Our data could be used as a benchmark for future work aiming at miRNA engineering of stress tolerance in transgenic crops, while these miRNAs could serve as diagnostic markers for stress conditions, as they can coordinate the regulation of multiple stress-signaling pathways as demonstrated in our study.

## Supplemental Information Available

Supplemental material is available online for this article.


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