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ORIGINAL RESEARCH

# miR171 Family Members are Involved in Drought Response in *Solanum tuberosum*

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**Abstract** MicroRNAs (miRNAs) are a group of small non-coding RNAs found in both animals and plants. miRNAs function as negative post-transcriptional regulators during plant development and in response to biotic and abiotic stress. In this work, we identified drought stress-related miRNAs from reads obtained from second-generation sequencing. This method is useful to determine miRNA expression profiles when genomic sequences are not available and to find putative miRNAs by aligning reads with sequences from miRBase. Here, we report the identification of a family of drought-responsive miRNAs, stu-miR171a, stu-miR171b, and stu-miR171c, from potato plants, their expression profiles upon drought stress, and their target mRNAs.

**Keywords** Deep sequencing · Drought stress · MicroRNAs · *Solanum tuberosum*

MicroRNAs (miRNAs) are a group of small non-coding RNAs found in both animals and plants. The mature miRNAs conserved across plants are usually 20–25 nucleotides (nt) in length and derived from stem-loop regions of approximately 70 nt RNA precursors by Dicer-like enzymes of the RNase III (Bartel 2004; Kim et al. 2005; Murchison and Hannon 2004; Jones-Rhoades et al. 2006).

The mature miRNA forms a multiple-protein nuclease complex called an RNA-induced silencing complex (RISC) with proteins including a key component Argonaute (AGO). Once incorporated into a RISC, the miRNA is situated to regulate the target genes by degradation of the mRNA through direct cleavage or by inhibiting protein synthesis. Plant miRNAs can bind to sequences on target mRNAs by exact or near-exact complementary base pairing and thereby direct cleavage and destruction of the mRNA (Rhoades et al. 2002; Chen 2005) or by repression of target mRNA translation (Jones-Rhoades et al. 2006). Thus, miRNAs function as negative post-transcriptional regulators.

Plants, which are sessile, have evolved a variety of protective mechanisms to overcome diverse abiotic environmental stresses such as severe changes in temperature, drought, and salinity (Zhu 2001a, b; Xiong et al. 2002; Xiong and Zhu 2002). Since abiotic stress disrupts various cellular functions, a rapid and extensive molecular defense mechanism both at the transcriptional and post-transcriptional levels is essential to recover from the abiotic stressed conditions. Transcription factors are master gene regulators that control many genes at a time and are involved in the abiotic stress response (Knight and Knight 2001; Yamaguchi-Shinozaki and Shinozaki 2006). Ever since the first plant miRNAs were cloned from *Arabidopsis thaliana* (Llave et al. 2002; Park et al. 2002; Reinhart et al. 2002), many miRNAs and their target genes have been identified and characterized in various plant species. The majority of miRNA target genes were found to encode various transcriptional factors or important functional enzymes and play important roles in plant development and response to various biotic and abiotic stresses.

The initial discovery of miRNAs and their involvement in plant growth and development led to the identification of stress-related miRNAs (Jones-Rhoades et al. 2006).

Direct evidence of miRNA involvement in plant abiotic stress comes from the identification of miR398, which

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targets two Cu/Zn superoxide dismutases (SODs). Study of the expression of Cu/Zn SODs during oxidative stress stemming from high salinity, drought, heavy metal content, light, and extreme temperature conditions found post-transcriptional control by miR398, indicating the role of miRNA-mediated regulation of SODs during abiotic stress (Sunkar et al. 2006).

Several other miRNAs were also found to be involved in the abiotic stress response (Sunkar and Zhu 2004; Sunkar et al. 2007). MiRNA159, which targets MYB transcription factors (MYB33, MYB65, MYB101, and MYB104), is reportedly induced by the stress hormone abscisic acid (ABA) under drought conditions (Reyes and Chua 2007).

Using miRNA microarrays, 14 miRNAs induced by high salinity, drought, and low temperature were identified from *Arabidopsis* (Liu et al. 2008). Of these 14 stress-inducible miRNAs, miR168, miR171, and miR396 are upregulated during salt, osmotic, and cold stresses, although their exact role in plant abiotic stress is not known (Liu et al. 2008).

In the last few years, second-generation sequencing methods such as Solexa and 454 sequencing have been developed for the purpose of inexpensive and high-throughput sequencing. These new methods have been proven to have high sensitivity and specificity. The Solexa sequencing by synthesis technology of Illumina is capable of generating more than 50 gigabases raw reads of 36 nt length per full run. Thus, they can be effectively used to measure the abundance of small RNA sequences in a sample.

Therefore, the expression profiling of miRNAs can be exploited using deep sequencing methods, which can be used to identify novel miRNAs.

In this work, we identified drought stress-related miRNAs from second-generation sequencing reads using a systematic method. This method efficiently revealed miRNA expression profiles by aligning sequencing reads against sequences from the miRNA database (miRBase). Therefore, this method can be used to determine miRNA expression profiles when genomic sequences are not available and find putative miRNAs by aligning reads with sequences from miRBase. Here, we report the identification of a family of drought-responsive miRNAs, stu-miR171a, stu-miR171b, and stu-miR171c, from potato plants, their expression profiles upon drought stress, and their target mRNAs.

## Materials and Methods

### Plant Materials and Drought Treatments

Potato plants (*Solanum tuberosum* subsp. Choobaek) were acquired from the National Institute of Highland Agriculture (NIHA) at the Rural Development Administration of Korea (RDA). Potato plants were cultured hydroponically in MS medium (MS salt, vitamin mix, 3% sucrose, pH 5.6~5.7) and maintained in a temperature-controlled culture room at 22°C under 16 h/8 h light/dark conditions. Drought conditions were simulated by air-drying or with 15% polyethylene glycol (PEG 6000 Duchefa Biochemie, the Netherlands) for various durations. Untreated plants were used as a control. Entire plants were immediately transferred into liquid nitrogen.

### Total RNA Isolation

Total RNA was extracted from control and drought stress-treated potato plants using Tri-Reagent (MRC, USA) according to the manufacturer's instructions.

### miRNA Identification

#### *Small RNA Isolation from Total RNA and Ligation of 5′ and 3′ Adapters*

Polyacrylamide gel electrophoresis (PAGE) was used to isolate small RNAs ranging in length between 20 and 30 0 nt from total RNA obtained from control and drought stress-treated potato plants. The isolated small RNAs were single-strand ligated with 3′ adapters (5′-P-UCGUAUGCCGUCUU CUGCUUGU-3′) and 5′ adapters (5′-GUUCAGAGUUCUA CAGUCCGACGAUC-3′) sequentially, followed by reverse transcription and amplification using polymerase chain reaction (PCR). The resulting products, "DNA Colony Template Library," provided templates for deep sequencing.

#### *Quality Control of "DNA Colony Template Library" and Deep Sequencing*

Before performing deep sequencing, eight template DNAs per time point were cloned into a TOPO plasmid for quality control. Quality control was performed by regular sequencing of the inserts and by checking the sizes of the small RNAs. For deep sequencing, the "DNA Colony Template Library" was diluted to 10 nM, and the flow cell was prepared. Deep sequencing was performed using an Ilumina Genome Analyzer (Fateris, Switzerland).

#### *miRNA Reference*

The miRNA database (http://www.mirbase.org/) containing previously known miRNA sequences from *A. thaliana*, *Glycine max*, *Oryza sativa*, *Populus trichocarpa*, *Saccharum officinarum*, *Sorghum bicolor*, *Zea mays*, etc., was used as a reference set for those potato miRNAs involved in drought stress.

### miRNA Northern Blot Analysis

To obtain mature miRNA expression profiles upon drought stress, Northern blot analysis of miRNAs was performed. Total RNA was loaded (40 μg per lane) and then resolved on a denatured 15% polyacrylamide gel. The gel was separated in two parts; one containing 5S rRNA was stained by ethidium bromide, and the other containing miRNAs was transferred to a Hybond-N+ nylon membrane (Amersham, USA). The membranes were then cross-linked with UV at 700 mJ/cm$^2$. DNA probes complementary to the miRNA sequences were end-labeled with [γ-$^{32}$P]ATP (3,000 Ci/mmol, EasyTide, Perkin Elmer, USA) using T4 polynucleotide kinase (Takara, Japan). Unincorporated [γ-$^{32}$P]ATP was removed by Sephadex G-25 column chromatography. Prehybridization and hybridization were carried out using ULTRA hyb®-Oligo Hybridization Solution (Ambion, USA) at 42 or 37°C. Blots were washed in 2× SSC/0.5% SDS.

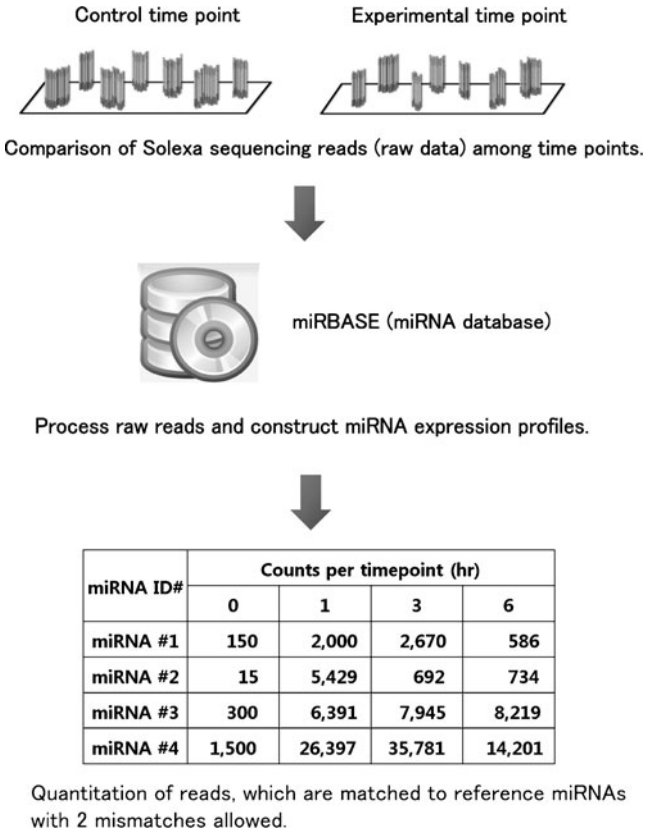
### Prediction of Putative Regulatory Targets of stu-miRNA

The National Center for Biotechnology Information (NCBI) GenBank nucleotide database was used to find the putative regulatory targets of the miRNAs. One can exploit the fact that plant miRNA sequences bind to their targets with perfect or near-perfect complementarity to predict potential targets for miRNA sequences using a computational approach (Rhoades et al. 2002; Jones-Rhoades and Bartel 2004). The number of allowed mismatches at complementary sites between miRNA sequences and potential mRNA targets was no more than two, and no gaps were allowed at the complementary sites.

## Results

### Identification of Drought-Responsive stu-miR171 Family Members

To identify miRNAs from potato plants upon drought stress, we first treated potato plants with drought stress at 0, 1, 3, and 6 h. Then, small RNAs ranging in size from 20 to 30 nt were obtained from total RNA using PAGE (see "Materials and methods"). After adapter ligation at the 5′ and 3′ ends of the small RNA samples from each time point, including the 0 h control sample, deep sequencing was carried out using the Solexa Sequencing System of Illumina. Then, the sequencing reads were compared between each time point. Small RNA sequencing reads that were deemed significant were then compared with the miRNA database to identify potato miRNAs involved in drought stress. Figure 1 shows how to construct the miRNA expression profiles and identify miRNAs using raw sequence reads produced by Solexa sequencing.




| miRNA ID# | Counts per timepoint (hr) | | | |
|---|---|---|---|---|
| | 0 | 1 | 3 | 6 |
| miRNA #1 | 150 | 2,000 | 2,670 | 586 |
| miRNA #2 | 15 | 5,429 | 692 | 734 |
| miRNA #3 | 300 | 6,391 | 7,945 | 8,219 |
| miRNA #4 | 1,500 | 26,397 | 35,781 | 14,201 |

Quantitation of reads, which are matched to reference miRNAs with 2 mismatches allowed.

**Fig. 1** Strategies for construction of miRNA expression profiles and identification of stu-miR171 family members using raw sequence reads of deep sequencing

Briefly, at each time point, including the 0 h control, adapter sequences were removed, and identical reads were counted. In the case in which the adaptor sequence was in the middle or at the beginning of the read, the entire read was removed. If the adaptor sequence was at the end of the read, however, then the adaptor sequence was simply trimmed out. In the second step, each read was aligned with the sequences from the miRNA database (miRBase). The proposed alignment algorithm was based on the Smith–Waterman algorithm. In the third step, miRNA expression profiles were constructed by calculating the sum of the read counts for each miRNA according to the alignment criteria (e.g., the length of the read equals the length of the miRNA sequence and the identity of the alignment with two mismatches allowed).

From the miRNA expression profile, members of the miR171 family, stu-miRNA171a, b, and c, were regulated by drought treatment (Table 1). Mature stu-miR171a contains a "UGAUUGAGCCGUGCCAAUAUC" sequence, whereas mature stu-miR171b and stu-miR171c contain "UUGAGCCGUGCCAAUAUCACG" and "UGAGCCGAACCAAUAUCACUC," respectively. Stu-miR171a, b, and c were 21 nt in length. The individual miRNA expression levels of these miRNAs upon drought stress were measured by Northern blot analysis.

**Table 1** Sequences and homologous miRNAs of stu-miRNA171a, b, and c

| | miRNA sequence | Homologous miRNAs |
|---|---|---|
| stu-miR171a | UGAUUGAGCCGUGCCAAUAUC | osa-miR171b, c, d, e, f<br>sbi-miR171a, b, d, I, k<br>zma-miR171d, e, I, j<br>ptc-miR171e, f, g, h, i<br>tae-miR171<br>vvi-miR171a, c, d, i<br>sly-miR171a<br>mtr-miR171d, g<br>bdi-miR171c<br>aqc-miR171a, b, d<br>crt-miR171<br>rco-miR171c, d, e, f |
| stu-miR171b | UUGAGCCGUGCCAAUAUCACG | ath-miR171b, c<br>ptc-miR171a, b<br>bna-miR171a, b, c, d, e<br>sly-miR171b<br>bra-miR171a, b, c, d<br>bol-miR171a<br>mtr-miR171f<br>rco-miR171a, b<br>zma-miR171b |
| stu-miR171c | UGAGCCGAACCAAUAUCACUC | osa-miR171h<br>sbi-miR171e<br>zma-miR171k, h<br>sbi-miR171f |

## Expression of miRNA171 Family Members Upon Drought Treatment

We analyzed stu-miRNA171a, b, and c expression upon drought stress treatment of potato plants by Northern blot analysis.

Accumulation of mature stu-miR171a upon air-drying decreased 1 h after stress treatment, followed by an increase thereafter. Similarly, accumulation of mature stu-miR171a upon 15% PEG 6000 treatment decreased 1 h after stress treatment followed by an increase up until 3 h and then constant maintenance thereafter (Fig. 2a).

Accumulation of mature stu-miR171b upon air-drying decreased 1 h after stress treatment, followed by recovery up to control levels until 3 h, and then a further increase up until 6 h after. However, 15% PEG treatment resulted in a slight decrease in accumulation of mature stu-miR171b until 6 h after stress treatment, followed by an increase to maximum level until 12 h after (Fig. 2b).

Accumulation of mature stu-miR171c upon air-drying treatment decreased 1 h after stress treatment then increased thereafter. This pattern was similar to that of stu-miR171a. However, accumulation of mature stu-miR171c upon 15% PEG treatment decreased 1 h after treatment followed by a

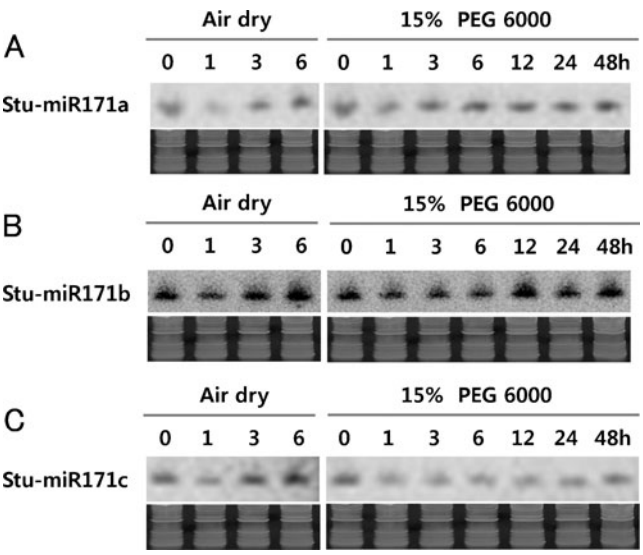



**Fig. 2** Expression of miRNA171a, b, and c (**a**, **b**, and **c**, respectively) upon drought treatment by Northern blot analysis. Forty micrograms of total RNA was loaded per lane. *Bottom panels* indicate ethidium bromide-stained RNA PAGE gel and serve as loading controls

**Table 2** Putative target mRNAs of stu-miR171a, b, and c

| miRNA | Accession no. | Target genes | Percent |
|---|---|---|---|
| stu-miR171a | XM_002336516.1 | *Populus trichocarpa* GRAS family transcription factor (GRAS58-63, 65), mRNA | 100 |
| | NM_130079.2 | *Arabidopsis thaliana* scarecrow transcription factor family protein (AT2G45160) mRNA | 100 |
| | NM_116232.4 | *Arabidopsis thaliana* scarecrow-like transcription factor 6 (SCL6) (AT4G00150) mRNA | 100 |
| stu-miR171b | XM_002327662.1 | *Populus trichocarpa* GRAS family transcription factor (GRAS65), mRNA | 95 |
| | XM_649620.2 | *Entamoeba histolytica* HM-1:IMSS protein phosphatase domain-containing protein, mRNA | 90 |
| stu-miR171c | XM_002513735.1 | *Ricinus communis* nodulation signaling pathway 2 protein, putative, mRNA | 90 |
| | XM_002306585.1 | *Populus trichocarpa* GRAS family transcription factor (GRAS 40, 42), mRNA | 90 |

slight increase until 48 h. Accumulation of mature stu-miR171c at 48 h after PEG treatment was identical as that of the 0 h control (Fig. 2c).

### Identification of Putative Targets for stu-miRNA171a, b, and c

The putative regulatory target genes for stu-miRNA171a, b, and c were predicted by identifying those mRNAs having perfect complementarity or near-perfect complementarity with allowed two mismatches (Table 2).

Of the three predicted targets for stu-miRNA171a, all were members of transcription factor gene families involved in developmental patterning or stress response. The targets for stu-miRNA171a include the GRAS family transcription factor.

Of the two predicted targets for stu-miRNA171b, one was a member of a transcription factor gene family involved in developmental patterning or stress response. The targets for stu-miRNA171b include the GRAS family transcription factor.

Of the two predicted targets for stu-miRNA171c, one was a member of a transcription factor gene family involved in developmental patterning or stress response. The targets for stu-miRNA171c include the GRAS family transcription factor.

## Discussion

In this paper, we identified drought stress-responsive miR171 family members, stu-miRNA171a, b, and c along with their putative target genes from *S. tuberosum*. A deep sequencing method, Solexa sequencing, which directly determines mature miRNA expression profiles, was applied. Specifically, this method is very useful in identifying miRNAs, especially when genomic sequences are not available, and finding putative miRNAs by aligning reads with sequences from miRBase.

Our approach used 18 to 26 nt small RNAs to construct the "DNA Colony Template Library," which was then used for miRNA identification. Thus, there were limitations in isolating precursors of those mature miRNAs. The sequences of complete precursor-miRNAs could be obtained after the potato genome project is completed.

It is known that miR171 is induced by abiotic stresses in the embryogenic and non-embryogenic callus tissues of *Larix leptolepis* and targets transcriptional factors belonging to the GRAS gene family, which are active in cell differentiation and development (Zhang et al. 2010).

The genes for GRAS family transcription factors, such as scarecrow (SCR), are targets for stu-miRNA171a, b, and c. GRAS transcription factors have been reported to be plant-specific proteins that participate in various developmental processes and stress responses such as drought stress (Ma et al. 2010).

The SCR gene was identified in *Arabidopsis* and is expressed specifically in root progenitor tissues of plant embryos as well as in certain roots and stem tissues. The SCR gene encodes a novel putative transcription factor and is required for asymmetric cell division in *Arabidopsis* root (DiLaurenzio et al. 1996). It is also known that the SCR gene is induced by abiotic stress such as salt and drought stresses (Ueda et al. 2002; Ma et al. 2010).

Further detailed study is required to disclose the modes of action of stu-miR171a, b, and c in drought stress-related regulation in *S. tuberosum*.

Since the number of available genomic sequence databases for potato plants is smaller than those for *Arabidopsis* and *O. sativa*, there is a limitation to the number of potato target genes. To obtain the complete sequences of precursors stu-miR171a, b, and c, the potato genome project must be completed or near-completed.

In addition to three drought-induced miRNAs, stu-miR171a, b, and c, we identified several more drought-stress responsive miRNA families in *S. tuberosum* by comparative deep-sequencing and sequence alignments. It includes families of miR159, miR164, miR166, miR390, miR395, miR397, miR398, miR408 and miR482. We are currently characterizing these miRNA families.

Although we found that stu-miR171a, b, and c were involved in drought stress by deep sequencing and

Northern blot analysis and also identified their putative target genes, further study is necessary to determine their actual defense mechanism against drought stress. One approach would be to obtain transgenic potato plants that overexpress stu-miR171a, b or c, which, when combined with microarray techniques, could result in more precise target genes of the stu-miR171 family. These efforts will elucidate the mode of action of stu-miR171a, b, and c during drought stress response.

For this, we currently are generating transgenic plants overexpressing stu-miR171a, b or c.

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