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IDENTIFICATION AND ANALYSIS OF DIFFERENTIALLY EXPRESSED miRNAs RELATED TO SEEDLING DEVELOPMENT IN THE *ARABIDOPSIS THALIANA* CYP71 MUTANT

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Abstract

CYCLOPHILIN71 (AtCYP71), a multidomain cyclophilin, has been demonstrated to participate in many aspects of development in *Arabidopsis thaliana*. Loss of AtCYP71 function results in pleiotropic developmental phenotypes including defects in shoot apical meristem (SAM), root apical meristem (RAM), leaf morphology as well as floral development. Previous studies have reported that microRNAs (miRNAs) play critical roles in plant development. However, to date, development-related miRNAs associated with AtCYP71 have rarely been reported. In our study, by employing small RNA (sRNA) and degradome sequencing, we surveyed development-related miRNAs and their targets in the wild type and *cyp71* mutant. We identified 253 known miRNAs in total through high-throughput sequencing of two sRNA libraries and degradome libraries constructed from 10-day-old seedlings of the wild type and *cyp71* mutant. Of these, 38 known miRNAs (16 increased and 22 decreased) were differentially expressed with normalized reads larger than 100. Via degradome analysis, 337 transcripts of 192 target genes targeted by 95 known miRNAs, including miR164, miR393, miR171, miR172 and miR824, were detected. Further functional analysis suggested that several identified targets, such as *NAC1*, AFB1, *SCL6* & *SCL23*, TOE2 and AGL16, were transcription factors that function in shoot meristem and lateral organ development together with nutrient metabolism. Overall, our results indicated that the disruption of AtCYP71 function contributes to altering the expression of some key miRNAs that target corresponding key genes that play critical roles in seedling development together with nutrient metabolism.

Key words: microRNA (miRNA); *Arabidopsis thaliana*; *cyp71*; High-throughput sequencing; Degradome analysis.

Introduction

Cyclophilins, which were initially discovered as cyclosporine A receptors, belong to a family of immunophilins and have been found in diverse organisms (Schreiber, 1991; He *et al.*, 2004). In plants, multiple cyclophilin members have been reported to be related to multiple functions, including protein folding and trafficking, protein degradation, mRNA processing, miRNA activity, RNA-induced silencing complex (RISC) assembly, chloroplast biogenesis and photosynthesis, and development (Krzywicka *et al.*, 2001; Fu *et al.*, 2007; Gottschalk *et al.*, 2008; Smith *et al.*, 2009; Iki *et al.*, 2012; Kang *et al.*, 2013). In *Arabidopsis*, AtCYP71 is a multidomain cyclophilin protein with a typical peptidyl prolyl cis-trans isomerase (PPIase) catalytic domain at its C-terminal and WD40 repeats at its N-terminal (He *et al.*, 2004; Li *et al.*, 2007). The latest research results show that PPIase activity is important for AtCYP71 functioning in chromatin remodeling during plant organogenesis (Lakhanpal *et al.*, 2021). Previous studies reported that loss of AtCYP71 function results in many phenotypic defects (Li *et al.*, 2007). In the *cyp71* mutant, plants displayed arrested apical meristem, delayed emergence of leaves, reduced root length and altered activity of root meristem and radial patterning. Compared with wild type, the SAM was broader in the *cyp71* mutant at 3 days after germination, but was much narrower in the *cyp71* mutant at 9 days after germination. Besides that, in the *cyp71* mutant, the three-cell layer organization of SAM was also disrupted. Some *cyp71* plants even displayed a more bushy stature, because of premature termination of the primary SAM. In addition, the rosette leaves of *cyp71* mutant plants were obviously distorted and not fully expanded, and failed to develop normal polarities (Li *et al.*, 2007). Moreover, AtCYP71 also participates in reproductive development in Arabidopsis (Li *et al.*, 2007; Li & Luan, 2011). In the *cyp71* mutant, the number of flowers was dramatically reduced, and the floral organ morphology was also changed, compared with wild type. Many studies have shown that the changes in miRNA expression usually lead to significant alterations in the normal morphogenesis and development of plants (Chuck *et al.*, 2009; Rubio-Somoza & Weigel, 2011), and that miRNAs seem to be more inclined to target transcription factors, most of which have potent functions in various biological processes (Sunkar *et al.*, 2012; Nova-Franco *et al.*, 2015). Up to date, however, no investigation on the identification of miRNAs associated with development has been reported in the *cyp71* mutant.

MiRNAs function as negative regulators in posttranscriptional gene regulation by repressing translation or guiding mRNA target degradation (Ambros *et al.*, 2003; Bartel, 2004; Jones-Rhoades *et al.*, 2006). In higher plants, miRNA genes are transcribed to primary miRNAs (pri-miRNAs) by RNA polymerase II. Next, the pri-miRNAs are processed into precursor miRNAs (pre-miRNAs),

which are characterized by a stem-loop structure. They were then cleaved into double-stranded miRNA/miRNA* duplexes with the help of hyponastic leaves 1 (HYL1), Dicer-like 1 (DCL1) and the C2H2-zinc finger protein SERRATE (SE) (Kurihara & Watanabe, 2004; Lee *et al.*, 2004; Kurihara *et al.*, 2006). The mature miRNA strand, which is methylated by HEN1 in the cytoplasm, is incorporated into the RISC containing an ARGONAUTE (AGO) protein (Yu *et al.*, 2005; Mallory *et al.*, 2008). The miRNA-RISC complex usually regulates targeted gene expression by cleaving targeted mRNA or inhibiting its translation in plants. Accumulating studies have evidenced that miRNAs are important regulators involved in the development and environmental adaptation of plants in many different ways (Xie *et al.*, 2015; Liang *et al.*, 2018; Song *et al.*, 2019; Wang *et al.*, 2019). For example, miR164 has been reported to control meristem development, separation of aerial organs and lateral root development through regulating *CUC1*/*CUC2* and *NAC1* expression (Rhoades *et al.*, 2002; Laufs *et al.*, 2004; Guo *et al.*, 2005). MiR165/166 affects adaxial/abaxial patterning of leaves by directing the cleavage of *PHB*, *PHV* and *REV* mRNA (Chen, 2005; Kidner & Martienssen, 2005). Overexpression of miR166a could accelerate vascular cell differentiation from cambial/procambial cells, resulting in a vascular system with dilated xylem tissue by guiding the cleavage of *ATHB15* mRNA (Kim *et al.*, 2005). MiR172 was reported to induce *CONSTANS* (*CO*)-independent *FT*, thus promoting photoperiodic flowering, and down-regulate the AP2-like target genes (including *AP2* and *TOE2*) via a translational inhibition mechanism, contributing to floral organ formation (Aukerman & Sakai, 2003; Jung *et al.*, 2007).

In this study, we carried out Illumina sequencing and bioinformatics analysis to detect and validate development-related miRNAs and their target genes. After construction and sequencing of two sRNA libraries as well as two degradome libraries. The expression profiles of a portion of significantly altered expressed miRNAs were examined, and their target genes were verified using quantitative reverse transcription-polymerase chain reaction (qRT–PCR) analysis.

Materials and Methods

Plant materials and growth conditions: Wild type (ecotype Columbia-0, Col-0) and the T-DNA insertion mutant line *cyp71* (accession number JP69.6C06; background, Col-0) of *Arabidopsis thaliana* were used for our investigation. The seedling samples were collected after the following procedures were performed. Firstly, seeds were sown on 1/2 MS (Sigma–Aldrich, M5524) medium [0.8% (wt/vol) agar, 0.8% (wt/vol) sucrose] supplemented with 1 × Gamborg's vitamins (Sigma-Aldrich, G1019) under surface-sterilized conditions. Next, they were stratified at 4°C for 3 d in the dark, and then moved to a growth chamber (BINDER, Germany) for ten days with a long-day photoperiod (16 h illumination of 150 μmol/m^2/s, and 8 h dark cycle) at 22°C.

RNA extraction: Total RNA was separated from 10-day seedlings of the wild type and *cyp71* mutant (Fig. S1) using the TRIzol reagent (Invitrogen, CA, USA) based on the procedure provided by the supplier. The quantity and integrity of total RNA were detected with an RNA 6000 Nano LabChip Kit and Bioanalyzer 2100 (Agilent, CA, USA) with RIN number > 8.0 (Supplementary Table 1). The quality of RNAs was also checked by agarose gel electrophoresis.

Table 1. Overview of data in the WT and cyp71 degradome libraries.

Type		WT	*cyp71*
Raw reads	Total	10,130,592	13,141,234
	Unique	4,104,290	4,699,372
ADT & Length[1]	Total	65,813	77,463
	Unique	27,968	31,413
Input cDNA[2]			
Mapped cDNA reads	Total	10,064,779	13,063,771
	Unique	4, 076,322	4,667,959
Coverd cDNA		29,851	30,068

[1]ADT & Length: Reads lacking ADTs and lengths <15 nt
[2]Input cDNA: *Arabidopsis thaliana* cDNA library; https://www.arabidopsis.org/portals/genAnnotation/gene_structural_annotation/annotation_data.jsp

Small RNA library construction and sequencing: Two sRNA libraries of the wild type (WT) and *cyp71* 10-day seedlings were constructed using an Illumina TruSeq Small RNA Preparation Kit (Illumina, San Diego, USA). Briefly, by using T4 RNA ligase, a 3p adapter (5'-TGGAATTCTCGGGTGCCAAGG-3') and a 5p adapter (5'-GUUCAGAGUUCUACAGUCCGACGAUC-3') were in turn linked to the small RNAs, and the corresponding cDNA was synthesized by reverse-transcription PCR. Next, the amplification products were cut from a 6% polyacrylamide Tris-borate-EDTA gel and purified. Finally, the purified DNA fragments were subjected to single-end sequencing on the Illumina HiSeq2500 platform (LC Sciences, Hangzhou, China). The raw data were processed with the Illumina Pipeline v1.5 software. The low-quality data and low-copy-number reads were removed. Then, distinct sRNAs ranging from 18 to 25 nucleotides (nt) were collected. The ACGT101-miR program (LC Sciences, Houston, Texas, USA) was then used for further data analysis (Li *et al.*, 2013).

Identification of known and novel miRNAs: After filtering out adaptors (ADTs), sequences with lengths shorter than 18 nt or longer than 25 nt, low-quality reads, mRNAs, repeats (http://www.girinst.org/repbase), Rfam (http://rfam.janelia. org) as well as junk data, the databases miRbase 21.0 and the Arabidopsis genome database (TAIR10) were used to map and identify miRNAs from the remaining reads. The alignment between the candidate sequences and the known Arabidopsis mature miRNAs was set within three mismatches (Yin *et al.*, 2008). The sequences that matched known Arabidopsis miRNAs were identified as known miRNAs. If the reads cannot map to known plant miRNAs and precursors but can align to the Arabidopsis genome, they will be considered as novel miRNA candidates. Furthermore, the UNAFold software (http://www.mfold.rna.albany. edu/?q =mfold/RNA-Folding-Form) was used to predict the potential pre-miRNAs by recognizing stem–loop structures from the Arabidopsis genome (Zuker, 2003; Meyers *et al.*, 2008). Only those that met the criteria that the reads had high minimal folding energy indexes (MFEIs) were regarded as novel miRNAs (MFEIs ≥ 0.9 for novel miRNAs) (Bonnet *et al.*, 2004).

Degradome library construction and deep sequencing: The two degradome libraries of wild type (WT) and *cyp71* 10-day seedlings were constructed according to the method described beforehand. (Ma *et al.*, 2010) with minor modifications. In brief, we first obtained enriched poly(A) RNAs, and then connected them to a 5p adapter containing an EcoP15 I recognition site at its 3p end. After reverse-transcription PCR, the linked RNAs were transformed into the corresponding cDNA. After EcoP15 I digestion and purification, the cluster analysis in an Illumina Cluster Station and sequencing using Illumina HiSeq2500 (LC Sciences, Hangzhou, China) was applied to the final product under the guidance vendor's recommended protocol. Lastly, the raw reads were acquired from Illumina's Pipeline v1.5 software. Then, the sequencing image analysis was analyzed by the Pipeline Fiercest Module, and base-calling was used by the Pipeline Bustard Moduleat.

Identification and annotation of targeted mRNAs: After sequencing, the clean reads were filtered from raw reads by eliminating adaptors and low-quality reads and were compared with the cDNA library in TAIR10. CleaveLand 3.0 was applied to align and analyze mapped cDNA reads with identified miRNAs (Addo-Quaye *et al.*, 2008; Addo-Quaye *et al.*, 2009). The alignment score of the miRNA and its target was calculated as 0.5 points for G:U pairs and 1 point for mismatched pairs or single nucleotide bulges. If there were mismatches within the core position (2-13 nt), the score was doubled. A low alignment score (≤4) was also considered. According to the number and abundance values of degradome sequences, all targets were categorized into five classifications (0, 1, 2, 3 and 4) (Addo-Quaye *et al.*, 2008; Addo-Quaye *et al.*, 2009; Ma *et al.*, 2010). Further, in order to reveal the potential effects of miRNAs and their targets, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) analyses were utilized.

Validation of miRNAs and their target mRNAs by qRT–PCR: In the previous step, we obtained the target genes of miRNAs through high-throughput sequencing. The qRT–PCR that was used for validation by showing the expression profiling of miRNAs and the target genes was described in our previous study (Zhao *et al*., 2021). In brief, 2 μg of total RNA used as a template was added with RNase-free DNase I (Promega). Stem–loop reverse transcription was carried out in a 10 μl system containing 1 μmol of the stem–loop reverse-transcription (RT) primer. The mixture was successively incubated at 70°C on ice for 5 minutes. Next, 5 × M-MLV Reaction Buffer, 25 U Recombinant RNasin® Ribonuclease Inhibitor, 0.4 mM dNTP mix and 200 U M-MLV Reverse Transcriptase (Promega) were added to the mixture. The final volume was up to 25 μl. The reaction was performed at 42°C for 1 h and 70°C for 15 min. 18S rRNA was used as the internal control.

To analyze the target genes, 2 μg of total RNA was treated with RNase-free DNase I (Promega) and used for cDNA synthesis with oligo (dT) primers and M-MLV Reverse Transcriptase (Promega).

The qRT–PCR was performed using SYBR Premix Ex Taq™ (Takara, Dalian, China) on a CFX Connect Real-Time System (Bio-Rad, CA, USA). The reaction parameters were 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, and 60°C for 30 s. The primers used in the qRT–PCR were listed in Supplementary Table 6. *Actin 2* was used as the internal control. In addition, all sample analyses were performed with 3 technical replicates, and relative expression levels were quantified by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Chi-square 2 × 2, Fisher's exact test and Log_2 comparison were performed on normalized data to determine miRNAs differentially expressed in WT and CYP71 libraries. Fisher's exact test and chi-squared 2 × 2 P values were adjusted by the Benjamini–Hochberg error detection rate (FDR). (Audic & Claverie, 1997; Benjamini & Yekutieli, 2001). The t-tests of miRNA and target genes were each performed using "Multiple unpaired t tests" of GraphPad Prism version 9.3.0 for windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

Data availability statement: The clean sequencing data of all samples have been submitted to the Sequence Read Archive (SRA) with the accession number PRJNA330596. Supplementary Tables 1 to 6 in this article are publicly available in the figshare repository: https://doi.org/10.6084/m9.figshare.21591633 .

Results

Data analysis of small RNAs from the WT and *cyp71* libraries: Two sRNA libraries of wild type and *cyp71* 10-day seedlings were constructed. A total of 13,222,000 and 12,496,935 raw reads were yielded through deep sequencing from wild type and *cyp71* mutant libraries (Supplementary Table 2), respectively. After removal of sequences with low quality, adapters, sequences less than 18 nt and larger than 25 nt in length, junk reads, Rfam, mRNA and repeats, we obtained 7,559,382 and 7,497,107 clean reads corresponding to 795,033 and 407,177 unique reads from the two sRNA libraries, respectively (Fig. 2 and Supplementary Table 2).

Fig. 3 and Supplementary Table 3 summarized the length distribution of the sRNAs in both libraries. The majority of sRNAs ranged from 20 to 24 nt in length. Among the unique sequences, the proportion of 24 nt sRNA was the most abundant, which indicated that the data of sRNA libraries obtained by the Illumina sequencing were reliable.

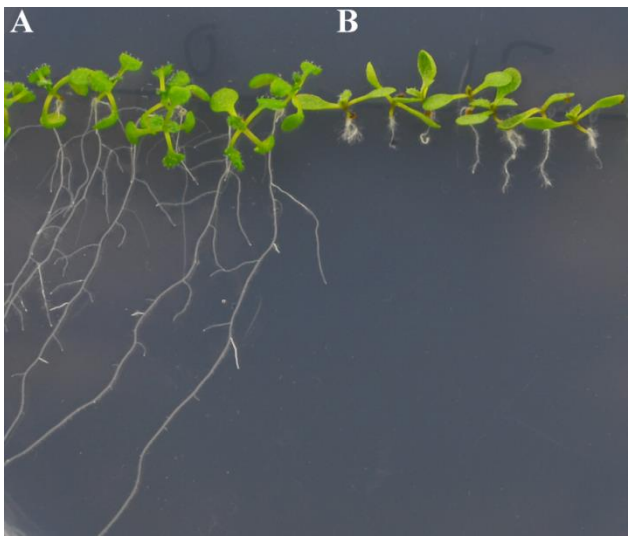

Fig. 1. Ten-day seedling phenotype of the wild type and *cyp71* mutant.
(A) Wild type (ecotype Columbia-0, Col-0); (B) T-DNA insertion mutant line *cyp71* (accession number JP69.6C06; background, Col-0).

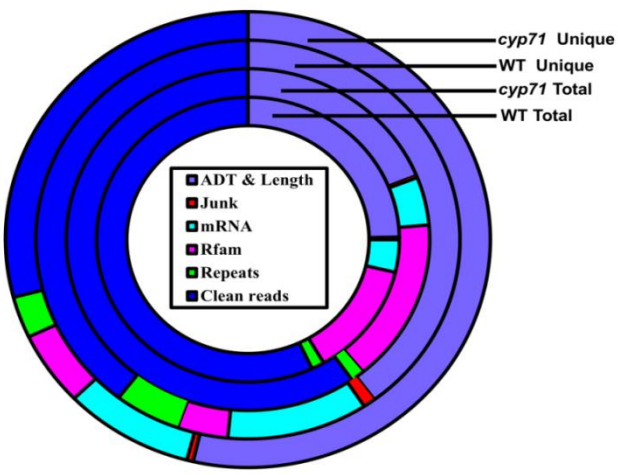

Fig. 2. Overview of sRNA sequences in the WT and *cyp71* libraries. *cyp71* Total and WT total represent the total clean reads of *cyp71* mutant and WT seedlings respectively. *cyp71* Unique and WT Unique represent a set of identical sequences among the total clean reads in *cyp71* mutant and WT seedlings, respectively. ADT & Length: Reads lacking adapters or lengths <18 or >25 nt. Junk: reads ≥2 N, ≥7 A, ≥8 C, ≥6 G, ≥7 T, ≥10 dimer, ≥6 trimer, or ≥5 tetramer. Rfam: Noncoding sRNAs except miRNAs. mRNA: mRNA fragments. Repeat: Prototypic sequences representing repetitive DNA.

Identification of known and potentially novel miRNAs: To filter known miRNAs, we compared the total clean reads with *A. thaliana* mature miRNAs in miRbase 21.0 and obtained 253 known miRNAs belonging to 118 miRNA families that were identified within two sRNA libraries (Supplementary Table 4). The member numbers varied widely between miRNA families (Supplementary Table 4). For example, miR169 was led with 9 members, leaving the miR156 family with 8 members. The abundance of the identified known miRNAs, as reflected in normalized data, showed great variation among families in both libraries (Supplementary Table 4). For example, miR158 presented the highest expression abundance with 65,229 reads in WT and 57,935 reads in *cyp71*, followed by miR165 and miR159. In addition, we also observed that in a certain miRNA family, some miRNA members were highly expressed; in contrast, some were expressed at low levels (Supplementary Table 4). For example, miR158, a miRNA family with high expression, has four members (ath-MIR158b-p5, ath-miR158b_L+1, ath-miR158a-5p and ath-miR158a-3p). The ath-miR158a-3p (64,276 reads in WT and 55,681 reads in cyp71) exhibited a high expression level, whereas only 21 reads of ath-MIR158b-p5 were detected in the *cyp71* library. A total of 237 miRNAs fell into these two libraries; 13 miRNAs were exclusive to the WT library, whereas 2 were found only in the *cyp71* library (Supplementary Table 4).

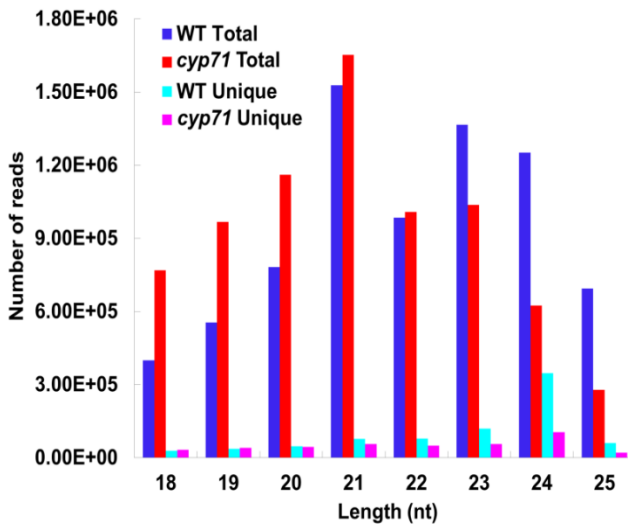

Fig. 3. Sequence length distribution of small RNAs in *cyp71* and wild type libraries.
cyp71 Total and WT total represent the total clean reads of *cyp71* mutant and WT seedlings respectively. *cyp71* Unique and WT Unique represent a set of identical sequences among the total clean reads in *cyp71* mutant and WT seedlings, respectively.

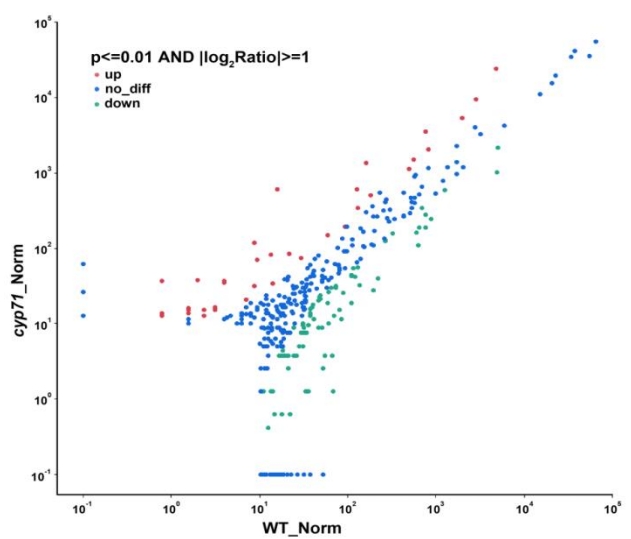

Fig. 4. Comparison of expression patterns of miRNAs identified between *cyp71* and wild type libraries. Each dot represents one miRNA. *cyp71*_Norm and WT_Norm represent normalized reads of each miRNA in *cyp71* mutant and WT seedlings, respectively.

In addition, 101 potentially novel miRNAs that met the criterion of plant miRNA identification were obtained. The majority of potentially novel miRNAs ranged in length from 21-24 nt. Among novel miRNAs, PC-5p-85928_10 was detected only in the *cyp71* library, and 9 novel miRNAs, including PC-3p-259851_3, PC-3p-74339_13, PC-3p-87881_10, PC-5p-259110_3, PC-5p-273637_3, PC-5p-327943_3, PC-5p-348301_3, PC-5p-58946_20, and PC-5p-93693_9, were found only in the wild type. Generally, the novel miRNAs were expressed at lower levels (normalized reads < 100), except for 4 potentially novel miRNAs, PC-3p-14416_183, PC-3p-54444_24, PC-5p-18237_138 and PC-5p-35882_51 (Supplementary Table 4).

Differential expression analysis of known and potentially novel miRNAs: Based on the criteria of highly significant variation (Fisher's exact tests and |log2 (*cyp71*/WT)| ≥ 1 and $p < 0.01$ from both Chi-square 2 × 2), 139 differentially expressed miRNAs (96 known and 43 potentially novel) were obtained (Fig. 4, Supplementary Table 4). Among them, 38 miRNAs (30 known and 8 potentially novel) were increased, and 101 miRNAs (66 known and 35 novel) were decreased. It is significant that 38 known miRNAs (16 increased and 22 decreased) were differentially expressed with more than 100 normalized reads. Among these, 11 miRNAs, e.g., ath-miR158b_L+1, were highly expressed (normalized reads ≥ 1000). Furthermore, our results showed that the expression of 13 miRNAs exhibited significant differences ($p<0.01$ and |log2 (*cyp71*/WT) |≥2) with normalized reads ≥ 100, such as ath-miR408-3p and ath-miR843 (Supplementary Table 4).

Targets of miRNAs: To study the function of the identified miRNAs, degradome sequencing combined with bioinformatics analysis was used to detect their targets. From the WT and *cyp71* degradome libraries, a total of 10,130,592 and 13,141,234 raw reads corresponding to 4,104,290 and 4,699,372 unique raw reads were found, respectively (Table 1). The results of BLAST analysis within the two libraries showed that 4,076,322 and 4,667,959 were cDNA mapped unique reads that were then aligned to the miRNAs using the Cleaveland 3.0 pipeline. Finally, 337 sequences of 192 target genes targeted by 95 known miRNAs were identified (Supplementary Table 5).

In the degradome analysis, our data showed that, in some cases, the same miRNA was responsible for the cleavage of both homologous and heterogeneous targets (Supplementary Table 5), suggesting that this certain miRNA may take part in a variety of biological activities. For instance, *ATHB8*, *ATHB9*, *ATHB14* and *ATHB15* (HD-ZIP protein family) were targeted by ath-miR165a,b-3p, whereas *TIE4* (*TCP INTERACTOR CONTAINING EAR MOTIF PROTEIN*), *AtMYB65* and *ATPD* (ATP synthase F1, delta subunit family protein) were targeted by ath-miR159c_R-3; additionally, transcripts of *TIR1*, *AFB1*, and *AFB2* (transport inhibitor reaction 1/auxin signaling F-BOX protein), which were targeted by miR393 a,b-5p_R-1, were detected in our degradome results (Supplementary Table 5). On the other hand, homologous or similar targets are typically targeted by homologous miRNAs. For example, both ath-miR172a,b and ath-miR172e-3p can cleave *AP2* or *TOE2* (*AP2*-like ethylene-responsive transcription factor *TOE* family), and *GRF* (*growth regulating factor*) genes including *AtGRF1*, *AtGRF3*, *AtGRF4*, *AtGRF7* and *AtGRF9*, were cleaved by both ath-miR396a-5p and ath-miR396b-5p.

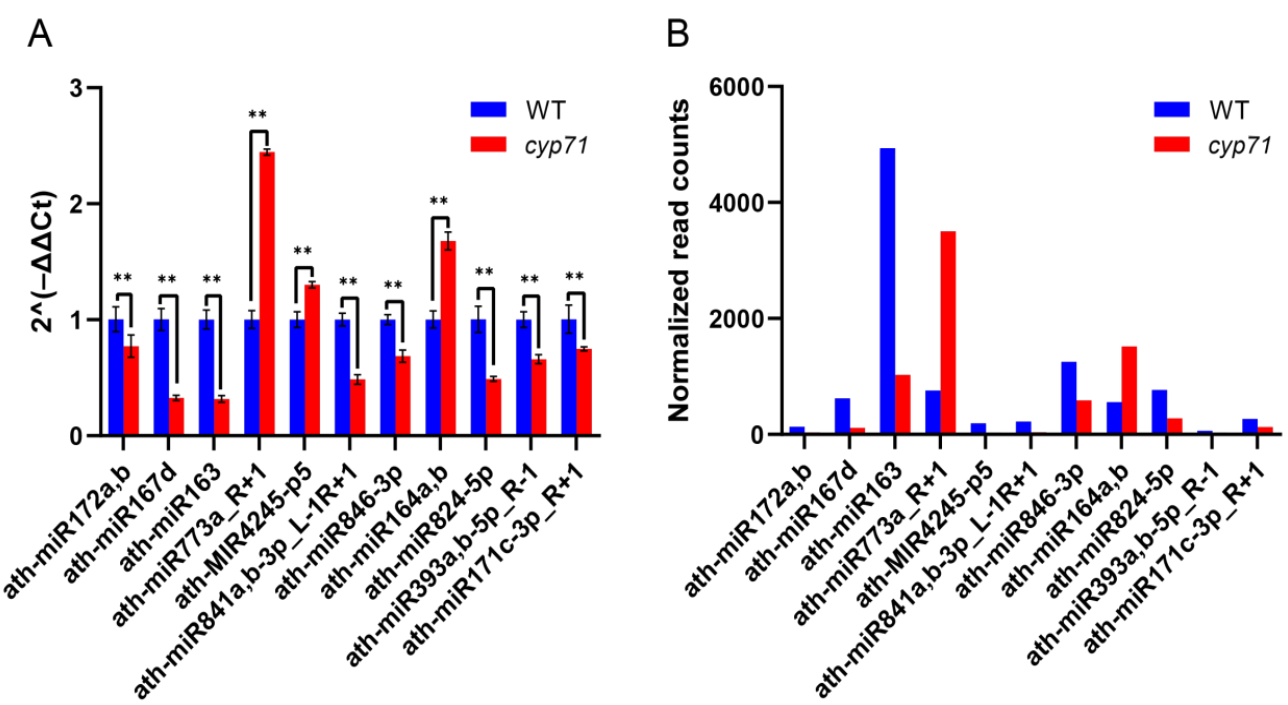

Fig. 5. qRT–PCR validation of some miRNAs differentially expressed in WT and *cyp71* libraries. (A) miRNA expression validated via qRT–PCR, '**' indicates significant differences (t-test q value <0.01, FDR approach) between WT and *cyp71*. (B) Normalized read number of selected miRNAs identified by high-throughput sequencing.

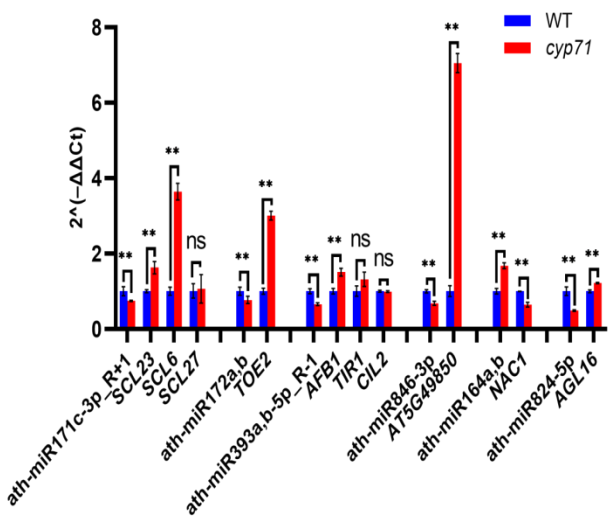

Fig. 6. Validation of targets in WT and *cyp71* degradome sequencing by qRT–PCR. Different groups of selected miRNAs and their target genes were separated with larger spacing. The ID prefix 'ath-miR' indicates the miRNA, followed by its target gene. '**' indicates significant differences(t-test q value < 0.01, FDR approach) between WT and *cyp71*. 'ns' indicates no significant difference between WT and *cyp71* mutant.

Additionally, 108 transcripts targeted by 40 differentially expressed miRNAs (37 known and 3 potentially novel miRNAs) were found by analyzing two degradome libraries (Supplementary Table 5). As a result, 31 targeted transcripts were exclusive to the WT degradome library, while 29 targeted transcripts just belonged to the *cyp71* degradome library.

One notable discovery was made during the study of the targeted transcripts of differentially expressed miRNAs. Several target genes encode proteins from different transcription factor families. These family members play crucial roles in various biological processes (Supplementary Table 5). For example, *NAC1* (*NAM/ATAF/CUC*) domain transcription factor), *SCL6*, *SCL23* and *SCL27* (*GRAS* gene family members), *TOE2* (*AP2*-like gene) and *AGL16* (*MADS-box* transcription factor), which were targeted by ath-miR164 (ath-miR164a,b and ath-miR164c-5p), ath-miR171 (ath-miR171a-3p, ath-miR171b-3p and ath-miR171c-3p_R+1), ath-miR172a,b, and ath-miR824-5p, respectively, participated in plant growth and development.

Validation of miRNA expression and the target genes by qRT–PCR: In order to verify our high-throughput sequencing results, we randomly selected 11 differentially expressed miRNAs to test using qRT–PCR. As shown in Fig. 5, although the fold of change in expression measured by qRT–PCR did not exactly correspond to the high-throughput sequencing results, the expression trends of 10 of 11 miRNAs were similar, except for ath-MIR4245-p5. Among those 10 miRNAs, the expression levels of ath-miR773a_R+1 and ath-miR164a,b were significantly up-regulated while the expression levels of other 8 miRNAs were significantly down-regulated in the *cyp71* mutant compared to the WT (Fig. 5). In addition, the expression levels of several miRNAs, including ath-miR397a, ath-miR408-3p, ath-miR408-5p and ath-miR857, were significantly up-regulated in the *cyp71* mutant.

Furthermore, the expression levels of 10 genes targeted by 6 miRNAs were assayed by qRT–PCR (Fig. 6). The transcriptional expression levels of *SCL23*, *SCL6*, TOE2, AFB1, AT5G49850 and AGL16 were significantly up-regulated in the *cyp71* mutant compared to the WT, while *NAC1* was decreased in the *cyp71* mutant, as shown by qRT–PCR.

As a result, it can be concluded that the data generated from high-throughput sequencing were reliable.

Discussion

The objective of our study was to illustrate the sRNA profiles of the wild type and *cyp71* mutant seedlings, particularly the miRNA expression profiles, which helped identify differentially expressed miRNAs with potential roles in development under the loss of function of AtCYP71. High-throughput sequence analysis in the present study indicated that most of the sRNAs ranged in length from 18-25 nt. These results are typical of Dicer-processed sRNA products and are consistent with the known range of 20-24 nt (Axtell, 2013) and 18-26 nt (Ghani *et al.*, 2018) for miRNAs . Molnar *et al.*, (2010) reported that it was in the root of grafted *Arabidopsis thaliana* that both the normalized total reads and non-redundant reads of 24 nt long small RNAs were more abundant than those of 21 nt long small RNAs (Molnar *et al.*, 2010). Actually, unique reads of 24 nt long small RNAs in both WT (Col-0) and *cyp71* seedlings were also more abundant than those of 21 nt long small RNAs in our study (Fig. 3).

However, total reads of 24 nt long small RNAs in both WT (Col-0) and *cyp71* seedlings were less abundant than those of 21 nt long small RNAs in this study, because whole seedlings of WT (Col-0) and *cyp71* mutant were collected as samples for extracting total RNAs including small RNAs. Previous studies have reported that three major classes of endogenous small RNAs, including miRNAs (the majority are 21 nt long), trans-acting siRNAs (ta-siRNAs, the majority are 21 nt long) and heterochromatic siRNAs (hc-siRNAs, the majority are 24 nt long), in plants, often exhibit spatial and temporal specificity (Breakfield *et al.*, 2012; Jeong *et al.*, 2013; Cheng *et al.*, 2017; Xu *et al.*, 2018). Furthermore, Xu *et al.*, (2018) reported that miRNAs were slightly more abundant than hc-siRNAs in cotyledons, juvenile leaves and cauline leaves (Xu *et al.*, 2018).

The known classic miRNAs, including miR164, miR171, miR172 and miR824, were detected. Besides, 101 potentially novel miRNAs were also obtained from the two libraries. Almost all potential new miRNAs exhibited lower levels of expression than known miRNAs. Notably, 38 known miRNAs (16 increased and 22 decreased) in the *cyp71* mutant were differentially expressed with more than 100 normalized reads. These results suggested that when compared with the wild type, the expression characteristics of miRNAs in the *cyp71* mutant were greatly affected.

Among the known miRNAs with significantly altered expression, several miRNAs were confirmed to function in regulating plant development. Herein, we found that ath-miR164a,b was up-regulated in the *cyp71* mutant. In plants, miR164, which is encoded by three genomic sites (*MIR164a*, *MIR164b*, and *MIR164c*), represses its target mRNAs of *NAC*-like transcription factors (including *CUC1*, *CUC2* and *NAC1*) via their degradation (Rhoades *et al.*, 2002; Laufs *et al.*, 2004; Guo *et al.*, 2005). *CUC1* and *CUC2* have been verified to participate in the formation of boundaries between emerging organ primordia and meristems (Laufs *et al.*, 2004; Nikovics *et al.*, 2006). *NAC1* is abundant in roots and seedlings, and overexpression of *NAC1* promotes the formation of lateral roots, whereas *NAC1* antisense expression reduces lateral root initiation (Xie *et al.*, 2000). Guo et al. provided evidence that *35S-MIR164* transgenic lines displayed lower lateral root numbers than wild type (Guo *et al.*, 2005). In 10-day seedlings, the *cyp71* mutant plants showed an emergence delay and a decrease in the numbers of lateral roots, compared with wild type (Fig. 1). We also noticed that the level of *NAC1* transcriptional expression was decreased in the *cyp71* mutant, as measured by qRT–PCR together with our degradome analysis. Thus, the up-regulation of ath-miR164a,b in the *cyp71* mutant might suppress the emergence and number of lateral roots. Although *cyp71* mutant seedlings exhibit obvious defects in the meristem and the development of meristem periphery organs, unfortunately, we did not find the transcripts of *CUC1* and *CUC2* in our degradome results.

TIR1/AFB2 (transport inhibitor reaction 1/auxin signaling F-BOX protein 2) take part in auxin perception and some aspects of auxin-dependent plant development. In *Arabidopsis*, miR393 reduced the expression of four TAAR genes (*AFB1*, *AFB2*, *AFB3* and *TIR1*) by guiding their mRNA cutting (Si-Ammour *et al.*, 2011). In our study, the expression of ath-miR393a, b-5p_R-1 was significantly decreased in the *cyp71* mutant, and the corresponding target transcripts of *TIR1*, *AFB1*, and *AFB2* were detected in our degradome results. The qRT–PCR results showed that the expression levels of *TIR1* and *AFB1* were up-regulated in the *cyp71* mutant. Overexpression in the form of miR393 of *TIR1* (*mTIR1*) enhanced auxin sensitivity and led to multifunction effects on plant development, including primary root growth inhibition, leaf phenotype alteration and delay in flowering (Chen *et al.*, 2011). A previous study reported that *TIR1* overexpression leads to a rise in *NAC1* transcript levels and an increase in the number of lateral roots; in contrast, the promotion effects of *TIR1* were countered by the *NAC1* transgene *35S*::anti-3' (Xie *et al.*, 2000). Thus, a significant drop in lateral root numbers in the *cyp71* mutant was observed because of the degradation of *NAC1* by the increased expression of miR164.

Three members of the GRAS gene family (*SCL6*, *SCL23* and *SCL27*), which are also named *AtHAM1*, *AtHAM2* and *AtHAM3*, have been verified to be associated with meristematic cell proliferation, polar organization and shoot branching (Rhoades *et al.*, 2002; Schulze *et al.*, 2010; Engstrom *et al.*, 2011). Wang *et al.*, (2010) reported that *SCL6*, *SCL23* and *SCL27* are the targets of miR171c in Arabidopsis (Wang *et al.*, 2010). We found a decreased accumulation of ath-miR171c-3p_R+1 in the *cyp71* mutant, implying that its target genes might be up-regulated. The qRT–PCR assay displayed that loss of AtCYP71 function resulted in enhanced expression of *SCL6* and *SCL23*. Schulze et al. reported that the accumulation of *SCL23* and *SCL27* transcripts in the peripheral and basal areas of the SAM is responsible for promoting cell differentiation at the periphery of shoot meristems and helping to maintain the polar organization (Schulze *et al.*, 2010). *SCL6* mRNA was specifically detected in the boundary region between developing organ primordia and the inner part of the meristem, which supports that *SCL6* is involved in axillary meristem development (Schulze *et al.*, 2010). We therefore concluded that the higher expression of *SCL6* and *SCL23* could contribute to promoting cell differentiation at the periphery of shoot meristems, leading to premature termination of the primary SAM and increased shoot branching in the *cyp71* mutant.

Proper flowering timing is essential for successful plant reproduction under constantly changeable environments. In *Arabidopsis*, miR172 and miR824 have been shown to play important roles in controlling flowering time (Aukerman & Sakai, 2003; Jung *et al.*, 2007; Hu *et al.*, 2014). In our study, the expression levels of ath-miR172a,b and ath-miR824-5p were down-regulated 4.1-fold and 2.94-fold in the *cyp71* mutant, respectively. Aukerman and Sakai reported that during the seedling stage, the temporal up-regulation of miR172 family members down-regulates the temporal expression of *AP2*-like targets (including *TOE1* and *TOE2*), thus promoting flowering (Aukerman & Sakai, 2003). Although a previous study evidenced that these genes were negatively targeted by miR172 through a translational mechanism (Aukerman & Sakai, 2003), we still observed that the cleavage product corresponding to *TOE2* was detected in our degradome analysis, as Kasschau et al. reported to detect the cleavage products of miR172 target sites by RT–PCR in *AP2*-like genes (Kasschau *et al.*, 2003). Furthermore, our qRT–PCR results showed that the transcript of *TOE2* was increased in the *cpy71* mutant. *AGAMOUS-LIKE16* (*AGL16*) belongs to the *MADS box* family of transcription factors, and is targeted for sequence-specific degradation by miR824. Hu et al. revealed that the *miR824*/*AGL16* module regulated the time of flowering repression by affecting *FLOWERING LOCUS T* (*FT*) in a long-day photoperiod (Hu *et al.*, 2014). Therefore, these results suggest that the decreased transcripts of miR172 and miR824 lead to the higher accumulation of *TOE2* and *AGL16* mRNAs, respectively, which contribute to flowering time repression in the *cyp71* mutant. Moreover, overexpression of *AGL16* transgenic plants resulted in incompletely expanded leaves (Kutter *et al.*, 2007), which is similar to the leaves of the *cyp71* mutant.

We also found that several miRNAs, including ath-miR397a, ath-miR408-3p, ath-miR408-5p and ath-miR857, were obviously up-regulated in the *cyp71* mutant. It was predicted and experimentally validated that miR397, miR408, and miR857 targeted the copper protein plantacyanin or other laccase copper protein family members and affected their transcription (Yamasaki *et al.*, 2007). Copper (Cu) is an important micronutrient essential for plant growth and development. Abdel-Ghany & Pilon (2008) observed that miR397, miR408, and miR857 were induced and that their targeted genes were negatively regulated, thus preserving Cu for the essential protein function when copper availability is limited. The cleavage products corresponding to *PLANTACYANIN* (*ARPN*) and *LACCASEs* (*LAC4* and *LAC7*), which were targeted by miR408, miR397 and miR857 respectively, were identified in our degradome analysis. The results of qRT–PCR analysis showed that the accumulation of *ARPN* and *LAC4* mRNAs was decreased in the *cyp71* mutant (data not shown). Thus, disruption of AtCYP71 function could also affect plant nutrient metabolism.

Conclusion

In conclusion, our results indicated that the disruption of AtCYP71 function contributes to altering the expression of some key miRNAs including miR164, miR393, miR171, miR172, and miR824, that target some key genes including *NAC1*, *AFB1*, *SCL6* & *SCL23*, *TOE2* and *AGL16* encoding transcription factors that play critical roles in shoot meristem and lateral organ development together with nutrient metabolism.

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