Original Research Article

Characterization and bioinformatics analysis of ptc-miR396g-5p in response to drought stress of Paeonia ostii

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ABSTRACT

Drought is one of the main abiotic stress factors affecting yield of Paeonia ostii. In this study, we conducted bioinformatics and differential expression analyses of P. ostii ‘Feng Dan’ ptc-miR396g-5p in leaf samples under different drought stress. ptc-miR396g-5p belongs to the miR396 family. Among the 271 plant species registered in the miRBase database, at least one miR396 member was found in 48 Angiospermae species, 3 in Gymnospermae species, and 1 in Pteridophyta. Mature sequence alignment showed that P. ostii ‘Feng Dan’ ptc-miR396g-5p had high sequence similarity with miR396 from other species. Secondary structure prediction showed that the precursor sequence of ‘Feng Dan’ ptc-miR396g-5p could form a stable stem-loop structure, and the mature sequence was located on the 5’ arm of the secondary structure. Phylogenetic tree analysis showed that ‘Feng Dan’ was closely related to 20 species such as Glycine max, Medicago truncatula, Populus trichocarpa, Citrus sinensis, Vitis vinifera, and Theobroma cacao. The predicted target gene of the ‘Feng Dan’ ptc-miR396g-5p encodes a Signal Transducer and Activator of Transcription (STAT) transcription factor. The negative correlation of expression between the miRNA and its target gene was confirmed by qRT-PCR. Our data indicate that ‘Feng Dan’ ptc-miR396g-5p’s expression decreases under drought, leading to an expression increase of the STAT transcription factor.

ARTICLE INFO

Keywords:
Drought response
Phylogenetic tree
miRNA secondary structure
miRNA target gene
qRT-PCR

1. Introduction

MicroRNA (miRNA) are a class of small noncoding regulatory RNAs encoded by endogenous genes that regulate gene expression. It inhibits the cleavage or translation of target genes by combining with Argonaute proteins (AGOs) to form an inducible silencing complex, thereby playing a role of regulatory effects at the transcriptional and post-transcriptional levels [1,2]. In recent years, miRNAs have been reported to widely exist in various plants and are involved in the regulation of plant growth and development, morphogenesis, flowering time, hormone signaling, and response to environmental stress [3–7]. Studies have shown that overexpression of miRNAs can improve the drought resistance of plants [8,9]. For instance, miR 394-overexpressing Arabidopsis plants are more resistant to drought stress than wild plants, indicating miR394’s important role in response to drought [10].

Tree peony (Paeonia suffruticosa Andrews.) belongs to section Moutan, genus Paeonia, and family Paeoniaceae [11], it has high ornamental value because of its rich flower color variation. Moreover, its roots can be used as medicine, and its seeds can be used for oil extraction, having high medicinal and edible value [12]. Tree peony is cultivated in many countries, such as China, Japan, France, the UK, the US, etc., of which China is the majority, and has a history of more than 1600 years. Studies have shown that tree peony seed oil is rich in essential unsaturated fatty acids, which can improve memory and enhance immunity, beneficial to human health [13–15]. In 2011, tree peony seed oil was approved by the Ministry of Health of the People’s Republic of China and officially entered the Chinese edible oil catalog as a new food resource [16,17].

Drought caused by water shortage has always been considered as one of the most destructive abiotic stresses. It affects a series of physiological and molecular responses of plants, and in severe cases, it can affect the normal growth and development of plants, resulting in a decrease in plant productivity [18,19]. The region with the most cultivars and the largest distribution area of tree peony is the central part of China, which has been in a dry climate for a long time. Severe water shortage will limit the cultivation area of tree peony, resulting in drooping leaves and smaller flowers, reducing the synthesis of organic matter, and finally
reducing seed yield [20,21]. In recent years, miRNAs in response to early flowering, fatty acid synthesis, chilling-induced dormancy release and copper stress in tree peony have been reported [22–25]. However, due to lack of research on miRNAs related to drought stress response, how miRNAs are involved in drought resistance in tree peony is unclear.

In this study, we analyzed the mature and precursor sequence of ptc-miR396g-5p in P. ostii ‘Feng Dan’ using bioinformatics, explored the evolutionary relationship of ptc-miR396g-5p in its gene family via the evolutionary conservation of miRNAs, and verified expression level of ptc-miR396g-5p by quantitative real-time PCR (qRT-PCR), to provide a reference for the research on the function of ptc-miR396g-5p in P. ostii. MiR396 is an ancient miRNA family that is highly conserved in plants. While several reports of miR396 are available in other plant species, this is the first study in tree peony.

2. Materials and methods

2.1. Experimental materials and drought treatment

In this study, healthy eight-year-old P. ostii ‘Feng Dan’ plants were used as the experimental material, which were planted in the experimental farm of Henan University of Science and Technology (112°24′52.05″E, 34°35′45.91″N). A gradual drought design was adopted, with irrigation being withdrawn on May 19, 2021. Leaves of control (CK, 28.44% soil moisture content), drought 9d (moderate drought, MD, 12.37% soil moisture content), 15d (severe drought, SD, 10.93% soil moisture content) and rehydration (RE, 29.82% soil moisture content), were wrapped in aluminum foil, immediately frozen with liquid nitrogen, and then stored in a −80 °C freezer for later use.

2.2. Target gene analysis of miR396 of P. ostii ‘Feng Dan’

Using the leaves of P. ostii ‘Feng Dan’ under CK, MD, SD, and RE treatments as the materials, transcriptome, miRNA and degradome sequencing were performed to identify differential expressed miRNAs and target genes. That is, differentially expressed miRNAs and differential expressed genes were intersected to get the miRNAs and genes coexpressed in different drought treatment, respectively. Then, the target genes of the coexpressed miRNAs were confirmed by degradome.

It was proved that ptc-miR396g-5p was one of the intersected different expressed miRNAs under drought treatments. The target gene prediction of ‘Feng Dan’ ptc-miR396g-5p was conducted using by degradome. PsRobot 1.2 (https://tools4mir4.org/software/target_prediction/psrobot/) and GSTAr 1.0 (https://github.com/MikeAxtell/CleaveLand4) were used for the target prediction to identify the miRNA binding sites. Sequences of miRNA hairpins and mature for other plant were downloaded from the miRBase (https://www.mirbase.org/) [26] and used for the blast and phylogenetic analysis.

2.3. Base conservation analysis of ‘Feng Dan’ ptc-miR396g-5p mature sequence

The software ClustalX 1.8 was employed for the alignment analysis of the mature sequences of ptc-miR396g-5p and members in the miR396 family obtained from the miRBase.

2.4. Characterisation of P. ostii ptc-miR396g-5p precursor sequences

The secondary structure of the P. ostii ptc-miR396g-5p precursor sequence and its stability were predicted by RNAfold (http://RNA.thi.unicv.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). To analyze the relationship, sequence characteristics, and degree of conservation of precursor sequences of miR396 family members, a phylogenetic tree consisting of P. ostii ‘Feng Dan’ and 52 other plant species, was constructed with MEGA 11.0 [27], using the neighbor-joining method and a bootstrap of 1000 [28].

2.5. Expression analysis of ‘Feng Dan’ ptc-miR396g-5p and its target gene by quantitative real-time PCR

miRNAs of ‘Feng Dan’ leaves were extracted using a miRcute Plant miRNA Isolation Kit (TIANGEN, Sichuan, China), and then cDNAs were synthesized using a miRcute Plus miRNA First-Strand cDNA Synthesis Kit (TIANGEN). A ubiquitin (UBQ) gene was used as reference [29]. A miRcute Plus miRNA qPCR Detection Kit (SYBR Green) (TIANGEN, including universal reverse primer) was used for qRT-PCR with a tailing method.

RNAs of ‘Feng Dan’ leaves were extracted using a RNAprep Pure Plant Plus Kit (Polysaccharides & Polyphenolics-rich) (TIANGEN), and then cDNAs were synthesized using a PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TakaRa, Shiga, Japan). A Tubulin-β gene was used as reference [30]. A TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (TakaRa) kit was used for qRT-PCR.

Three replicates were included for each sample respectively. Primers used for qRT-PCR are listed in Appendix Table 1. The expression levels of ptc-miR396g-5p and its target gene were calculated using the formula of 2−ΔΔCt [31]. Statistical analysis was performed using Excel and SPSS 21.0.

3. Results

3.1. Distribution of plants miR396

There were 271 plant species registered in the miRBase database (version 22.1), according to the search results, 52 plant species contained miR396 family information (Appendix Table 2). Along with ‘Feng Dan’, these species belong to Angiospermae, Gymnospermae, and Pteridophyta. Within Angiospermae, 38 were from Dicotyledoneae, and 11 were from Monocotyledoneae. Also, there were 3 species from Gymnospermae and 1 species from Pteridophyta. These 53 species were distributed in 26 orders and 27 families. Comparisons showed that the length of precursor sequence of miR396 differed greatly among different species, with the shortest sequence found in Zea mays (74 nt) and the longest in Citrus clementina (277 nt).

3.2. Base conservation analysis of ‘Feng Dan’ ptc-miR396g-5p mature sequence

Alignment analysis (Fig. 1) showed that ‘Feng Dan’ ptc-miR396g-5p shared the same sequence with the ones in Picea abies (miR396b/c/i/l/k/l/m/n/o/p/q/r/s/t), Populus trichocarpa (miR396g-5p), and Theobroma cacao (miR396d). When compared to Asparagus officinalis (miR396a), Cucumis melo (miR396e), Eugenia uniflora (miR396a-5p), Malus domestica (miR396f), Citrus sinensis (miR396e-5p), Picea abies (miR396f), Pinus densata (miR396e), Amborella trichopoda (miR396d), etc., ‘Feng Dan’ ptc-miR396g-5p exhibited only one base difference. When compared to Acacia auriculiformis (miR396), Glycine max (miR396e/h), Cynara cardunculus (miR396h), Zea mays (miR396g-5p/h), etc., ‘Feng Dan’ ptc-miR396g-5p exhibited two to four base differences. These results indicate that the mature sequence of ptc-miR396g-5p is highly conserved across species.

3.3. Characterisation of ‘Feng Dan’ ptc-miR396g-5p precursor sequence

When the secondary structure of the precursor sequence of ‘Feng Dan’ ptc-miR396g-5p was predicted using the online software RNAfold, the minimum folding free energy of ptc-miR396g-5p was predicted to be −42.00 kcal/mol. Meanwhile, the mature sequence bases are located on the 5‘ arm, which can form a stable stem-loop structure (Fig. 2).

As showed in Fig. 3, the phylogenetic tree can be divided into 6 groups, with ‘Feng Dan’ grouping with Glycine max (miR396e/h), Medicago truncatula (miR396c), Populus trichocarpa (miR396f), Citrus
Fig. 1. Multiple sequence alignment of mature sequence of miR396 gene family. ‘Feng Dan’ was in the second column, marked with a horizontal red box, *Picea abies* (miR396b/c/i/k/l/m/n/o/p/q/r/s/t) and *Populus trichocarpa* (miR396g-5p) were marked with a vertical red box.

Fig. 2. Secondary structure of ‘Feng Dan’ ptc-miR396g-5p precursor sequence. The mature sequence was located on the 5’ arm, marked with a red line.
This target gene encodes a Signal Transducer and Activator of Transcription (STAT) transcription factor. The predicted cleavage site of *ptc-miR396g-5p* on the target gene is shown in Fig. 4. *ptc-miR396g-5p* binds to the 3′ untranslated region (UTR) of the target gene, and the cleavage site is on 1082 bp of the target gene.

3.5. Quantitative real-time PCR differential expression analysis of *ptc-miR396g-5p*

qRT-PCR expression analysis was performed with the leaves of ‘Feng Dan’ under drought stress. As showed in Fig. 5 (A), under drought stress, the expression of *ptc-miR396g-5p* decreased: the CK and RE samples had the highest expression level, followed by the SD and MD samples. In contrast, the target gene expression increased under drought stress, with the highest level found in SD, followed by MD, RE, and CK. This indicates that *ptc-miR396g-5p* and target gene were differentially expressed under drought stress, and there was an inverse regulatory correlation between *ptc-miR396g-5p* and its target gene psu.T.00022975.

The expression dynamics of the miRNA and its target gene was compared between the RNA sequencing data and qRT-PCR (Fig. 5 B and C). When compared to the control, both methods showed that the miRNA expression decreased under drought stress, while its target gene expression increased. In the recovery phase, the target gene expression decreased to a level similar to the control as showed by the data from both RNA sequencing and qRT-PCR. Discrepancy was found in the recovery phase for the miRNA expression, with qRT-PCR showing
expression level similar to the control and RNA sequencing indicating a lower level.

4. Discussion

miRNAs are a class of non-coding small RNA molecules that widely exist in plants and often play an important role in plant growth, development and response to stress. In tree peony, a species having ornamental, oil, and medicinal values, miRNAs involved in early flowering [22], fatty acid biosynthesis [23], chilling induced dormancy [24], and copper stress [25] have been recently identified. Here, we report a ptc-miR396g-5p that is responsive to drought. Under stress, such as drought [32,33] and high temperature [34,35], plants will defend against environmental challenges by activating a variety of defense mechanisms, at physiological, biochemical, and molecular levels, including miRNA-mediated regulation.

The newly discovered tree peony miRNA belongs to the miR396 family. Members of miR396 were first cloned by Jones-Rhoades et al., in Arabidopsis and rice in 2004 [36]. Since then, more miR396 family members have been discovered, and they had been found to play important roles in plants. Among the registered species in the miRBase database, miR396 was present in 48 Angiospermae, 3 Gymnospermae and 1 Pteridophyta. There are 172 miR396 precursor sequences in the miRBase database, and their sequence lengths are unevenly distributed, ranging from 74 to 277 nt. The length of the precursor sequence of ‘Feng Dan’ ptc-miR396g-5p is 95 nt, and the length of its mature sequence is 21 nt. The precursor sequence can form a typical stem-loop structure, which meets the standard of a miRNA. In addition, alignment of plant miR396 mature sequences in this study showed that ‘Feng Dan’ ptc-miR396g-5p has high similarity with miR396 from other species, substantiating that miR396 is highly conserved as previously reported [37].

Growth-regulating factors (GRFs) are generally considered to be the target of miR396 [38]. The GRFs are plant-specific transcription factors defined by the presence of a WRC (Trp, Arg, Cys) domain involved in DNA-binding and a QLQ (Gln, Leu, Gln) domain involved in protein-protein interactions. In Arabidopsis, seven out of the nine GRFs (GRF1-4 and GRF7-9) are post-transcriptionally repressed by miR396 [39–42]. Studies have shown that miR396 and its target GRF can regulate not only cell proliferation, cell division [43], embryogenesis [44], growth, and development [43–45], but also biotic and abiotic stresses [46–48]. Studies have shown that miR396/GRF3 can regulate the leaf size and organ longevity of Arabidopsis thaliana [49]. When the precursor of the Populus trichocarpa ptc-miR396c, was transformed into tobacco, down-regulated expression of GRF was observed, which ultimately affected the floral organ development of tobacco [50]. This Populus trichocarpa ptc-miR396c shares the identical mature sequence with ath-miR396b. In soybean, 20 GmGRFs are targeted by seven gma-miR396s (gma-miR396a/b/c/e/h/1/k) [38].

Another kind of miR396 target gene belongs to the basic helix-loop-helix (bHLH) transcription factor [51]. It has been reported that miR396 repress bHLHs to regulate Arabidopsis petal size and root growth in seedlings [52,53]. In Chinese yam, potential targets include genes that are annotated as transcription factor (B3 domain-containing protein ABI3VP1, targeted by miRNA396b), and involved in transport and catabolism processes (IST1-like protein IST1, targeted by miR396a-5p and miR396b). Additional targets are receptor expression-enhancing protein 1 (REEP1, targeted by miR396a-5p and miR396b), photosystem II oxygen-evolving enhancer protein 2 (pshp, targeted by miR396a-5p and miR396b), and fatty acid biosynthesis (acytly-CoA carboxylase biotin carboxyl carrier acceB, targeted by miR396b) [54].

Due to the very limited genomic resources available in tree peony, only one target gene was found for ptc-miR396g-5p. The target gene encodes a Signal Transducer and Activator of Transcription (STAT) transcription factor, which belongs to a unique family of DNA-binding proteins that usually respond to various extracellular cytokine and growth factor signals. At present, this protein has been studied more in animals than in plants. STAT pathway is connected upstream with Janus kinases family protein and capable of integrating inputs from different signaling pathways. Notably, STAT3 and STAT5 are involved in cancer progression whereas STAT1 plays opposite role by suppressing tumor growth [55]. The available GO and KEGG functional annotations in plants show that STAT might be related to ethylene biosynthetic process. Under drought stress, we found that the transcriptomic expression of ptc-miR396g-5p significantly increased, while the opposite was observed for the STAT target gene. This is consistent with the regulatory relationship of most miRNAs discovered so far. Our data suggest that by its expression decrease under drought, ‘Feng Dan’ ptc-miR396g-5p regulates drought response via increasing the transcriptomic expression of the STAT transcription factor.

The qRT-PCR results largely correlated with the RNA sequencing data, with only one discrepancy found in the miRNA expression during the drought recovery phase.

miR396 has been found associated with drought-stress responses in several studies. In Arabidopsis, miR396 is upregulated due to water stress, and overexpression of miR396 in transgenic plants results in decreased stomatal density, transpiration, and improved plant drought tolerance [56]. However, there are opposite results in some other plants. miR396 was down-regulated by drought in rice and grapevine [57,58], similar to our results in tree peony. Overexpression of miR396c reduced plant tolerance to salt and alkali stress in rice [59]. Under water stress, tomatoes with down-regulated expression of miR396 exhibit reduced transpiration and a disproportionate decrease in photosynthetic rate, resulting in higher water use efficiency in plants [60]. When more tree peony genomic resources become available, it is likely that additional target genes may be found for ptc-miR396g-5p.

5. Conclusions

miR396 family members are found in Angiospermae, Gymnospermae and Pteridophyta. The ‘Feng Dan’ ptc-miR396g-5p is highly conserved compared to other species. As expected, the precursor sequence of ptc-miR396g-5p forms a stable stem-loop structure, and the mature sequence is located on the 5’ arm of the secondary structure. Under drought stress,
the expression of ‘Feng Dan’ ptc-miR396g-5p decreased and its target gene’s expression increased, indicating a negative regulatory correlation consistent with the regulatory relationship commonly found in miRNAs.

**Funding**

This work was supported by the Outstanding Youth Fund Project of Natural Science Foundation of Henan Province (202300410119); the Colleges and Universities Science and Technology Innovation Talent Support Plan of Henan Province (22HASTIT036), the National Natural Science Foundation of China (U1804233), and the Central Plains Academics of Henan Province, China (212101510003).

**Credit author contribution statement**

Lili Guo: contributed to the conceptualization, investigation, supervision, writing-review & editing, and funding acquisition. Jiajia Shen: contributed to the material preparation, methodology, data curation, formal analysis, and writing original draft. Chenjie Zhang: contributed to the investigation, methodology, and software. Qi Guo: contributed to the supervision and data curation. Haiying Liang: contributed to the formal analysis and writing-review & editing. Xiaogai Hou: contributed to the supervision, and writing-review & editing. All the authors have read and agreed to publish the version of the manuscript.

**Declaration of competing interest**

The authors declare that there are no conflicts of interest.
Table 1
Primers for qRT-PCR.

| Primers Sequences (5′-3′) |
|---------------------------|
| ptc-miR396g-5p (Forward) CGGTTCCAGGGCTTTCTGTACT |
| U6Q (Forward) TGGCAGAAAAAGGGTTTCTGAC |
| psu.T.00022975 (Forward) GGCAACGAGTGCAAGAATCA |
| psu.T.00022975 (Reverse) TGGAAAAGGCTATAGGAGGA |
| Tubulin-β (Forward) TTGAGAAGGGCAAGGATGT |
| Tubulin-β (Reverse) ACCAGAAAAGCAAGCAGC |

Table 2
Distribution of miR396 in plant species.

| Species | Abbreviation | Classification |
|---------|--------------|----------------|
| Acacia auriculiformis | aau | Angiospermae | Dicotyledoneae | Leguminales | Leguminosae |
| Acacia mangium | amg | | | | |
| Glycine max | gma | | | | |
| Lotus japonicus | lja | | | | |
| Medicago truncatula | mtr | | | | |
| Arabidopsis lyrata | alr | Cruciales | Cruciferae |
| Arabidopsis thaliana | ath | Cruciales | Cruciferae |
| Brassica napus | bna | | | | |
| Brassica rapa | bra | | | | |
| Camelina sativa | cas | | | | |
| Avicennia marina | ama | | | | |
| Aquilegia caerulea | aqc | | | | |
| Amborella trichopoda | air | | | | |
| Bruguiera cylindrica | bcy | | | | |
| Bruguiera gymnorrhiza | bg | | | | |
| Cynara cardunculus | cca | | | | |
| Citrus clementina | ccl | | | | |
| Citrus sinensis |csi | | | | |
| Cucumis melo | cme | | | | |
| Carica papaya | cpa | | | | |
| Digitalis purpurea | dpr | | | | |
| Eugenia uniflora | eun | | | | |
| Fragaria vesca | fve | | | | |
| Malus domestica | mdm | | | | |
| Prunus persica | ppe | | | | |
| Gossypium hirsutum | ghr | | | | |
| Hesperis matronalis | hbr | | | | |
| Manihot esculenta | mes | | | | |
| Ricinus communis | rcm | | | | |
| Linum usitatissimum | lus | | | | |
| Nicotiana tabacum | nta | | | | |
| Solanum lycopersicum | sly | | | | |
| Solarum tuberosum | stu | | | | |
| Paeonia ostii | ptc | | | | |
| Populus trichocarpa | ptc | | | | |
| Salvia sclarea | sol | | | | |
| Theobroma cacao | tcc | | | | |
| Vitis vinifera | vvi | | | | |
| Asparagus officinalis | asf | Monocotyledoneae |
| Aegilops tauschii | ata | | | | |
| Brachypodium distachyon | bdi | | | | |
| Festuca arundinacea | far | | | | |
| Oryza sativa | osa | | | | |
| Sorghum bicolor | sbi | | | | |
| Saccharum officinarum | sof | | | | |
| Saccharum sp. | sop | | | | |
| Triticum aestivum | tae | | | | |
| Zea mays | zma | | | | |
| Vriesea carinata | vca | | | | |
| Picea abies | pab | Gymnospermae | Coniferopsida |
| Picea densata | pde | | | | |
| Pinus taeda | pta | | | | |
| Selaginella moellendorffii | smo | Pteridophyta | Lycopsida |

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