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Original Article

Exploring the Effect of Methyl Jasmonate on the Expression of miR160 and miR166 and their Targeted Genes of Ajowan (*Trachyspermum ammi* (L.) Sprague) Medicinal Plant

Running Title: Effect of Methyl Jasmonate on the Expression of genes of ajowan

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ABSTRACT

Methyl jasmonate (MeJA) is a regulator that mediates the biosynthesis of secondary metabolism compounds. Plant microRNAs (miRNAs) play important roles in post-transcriptional gene regulation of plant growth, hormone signaling, and stress response. To explore the possible role of miRNAs in regulating the MeJA signaling pathway, here we investigated the effects of MeJA treatment on the expression of candidate miRNAs and their targets in *Trachyspermum ammi*. The study aimed to reveal the function of miR166 and miR166 in the controlled pathway of MeJA. To achieve our goal, two MeJA levels (0 and 100 mM) were conducted at four-point times (0, 6, 12, and 24 h). The findings elucidated that pri-miR160 and pri-miR166 increase in response to MeJA. This suggested that pri-miR160 and pri-miR166 are relevant to hormone transmission. This study suggested that monitoring microRNA expression in response to MeJA plays a significant role in *T. ammi* and highlights the co-expression regulatory networks on the miR160/166 target genes in coordinating growth with environmental factors. However, to better understand how the response MeJA is regulated by microRNAs, further analysis of JA pathway biosynthesis and signaling events is required.

Keywords: MeJA, miRNAs, RT-qPCR, *Trachyspermum ammi*.

Abbreviation: Methyl jasmonate; MeJA, MicroRNAs; miRNAs

INTRODUCTION

MicroRNAs (miRNAs) are small 19–22 nucleotide sequences of non-coding RNAs that play important roles in regulating gene expression [1]. Many miRNAs regulate plant metabolism, growth, biosynthesis of secondary metabolites, and response to many biotic and abiotic factors [2]. They fluctuate due to many internal and external elicitors. For instance, the plant hormone methyl jasmonate (MeJA), salicylic acid (SA) and abscisic acid (ABA) treatment elicits have been reported to regulate stress-responsive miRNAs [3].

Jasmonic acid (JA) is an important signal molecule and functions in a lot of biological processes including growth inhibition, senescence, plant defense, and secondary mechanisms [4]. They have induced plant secondary metabolites such as terpenoids, alkaloids, and phenylpropanoids [5]. MeJA had also been used to enhance the secondary metabolites production by eliciting defense responses in *Amomum villosum*, *Euphorbia pekinensis*, and *Hyoscyamus niger* [6,7,8]. In *Taxus chinensis*, distinct changes in miRNA profiles were reported in response to MeJ [9]. Several miRNAs involved in MeJA mock treatment have been identified in the accumulation of terpenoid biosynthesis, they are differentially expressed in *Euphorbia kansui* during MeJA treatment and significantly increased the accumulation of terpenoid biosynthesis [1]. In *Euphorbia kansui*, the miR169, miR156, miR166, miR171, and miR395 family in terpenoid biosynthesis, were regulated under MeJA treatment [1]. In addition, miR166 was suggested to regulate terpenoid backbone biosynthesis, sesquiterpenoid, and triterpenoid biosynthesis in *Chlorophytum borivilianum* [10], which indicated that miRNAs are key molecular determinants.

The miR160 family in plants targets the Auxin response factors (ARFs) involved in the auxin signaling pathways [11]. It is also known to be involved in the growth and development of plants. miR160-ARF10/16 also regulated the terpenoid indole alkaloid biosynthesis pathway [12]. In *Catharanthus roseus*, a significant upregulation in miR160 after MeJA treatment and a significant downregulated of *CrARF10/16*, and *CrARF17* (targeted by miR160) were observed after MeJA-treatment [12]. Downregulation of miRNA396b in *Catharanthus roseus* caused a significant *increase* in the expression of ARF16 and repressed expression of terpenoid indole alkaloids pathway genes under MeJA treatment [12]. In *Taxus chinensis* L MiRNA156 and miRNA168 expression levels were downregulated and in *Lycoris aurea* miRNA408 was upregulated after MeJA treatment [13, 14].

Ajwain (*Trachyspermum ammi*) is a medicinal plant belonging to the Apiaceae family. *T. ammi*, as a medicinal herb, is distributed in Asia and native to Iran where grows naturally in several provinces [15]. *T. ammi* seeds have various important medicinal properties such as antioxidant, antifungal, analgesic, antihypertensive, antispasmodic, antitussive, antiparasitic, and anthelmintic [16]. Ajowan seeds include high yields of essential oils with valuable main monoterpenes such as high thymol content. The main constituents of the essential oils of ajwain were thymol, γ-terpinene, and p-cymene (85%) [17]. The secondary metabolites produced by ajowan not only play an important role in sustainable pest management, but they have also been used as drug components to treat various diseases [18]. So, applying an approach to recognize their mode of biosynthesis pathway and regulation has become crucially important in plant species. Significant attempts via advanced molecular biology research were able to be implemented to detect trends that might be the gene (s) involved in the biosynthesis of secondary metabolites in plants [19].

Due to the absence of any availability of genomics and the lack of any information considering miRNAs in *T. ammi*, miR160 and miR166, two conserved miRNAs, were selected as the potential miRNAs regulating the terpenoid backbone biosynthesis biosynthesis pathway. In this study, we further investigate the expression of these two stress-responsive miRNAs and their targets following MeJA treatment. Analytical and functional characterizations of these stress-responsive miRNAs and their target genes allow us to determine the miRNA-mediated mechanisms in *T. ammi* upon MeJA treatment.

MATERIAL AND METHODS

Plant Material and Treatments

The ajwain seeds (Arak ecotype) were obtained from Pakan Company (Isfahan, Iran). The seeds were carefully cleaned and then uniform, healthy seeds of the same size were selected for planting and were then cultivated in pots (10 cm diameter × 10 cm height) that were filled with loam clay sand texture and soil compressed within each pot to provide a soil surface similar to the field. Soil electrical conductivity (EC) and pH during entire plant growth were kept at 2.5 μS/cm and 8.5, respectively. The seeds were maintained in a growth chamber (25 °C, 16 h photoperiod, and light intensity of 70% μmol m^{-2} s^{-1}). When the plants reached the 3-leaf stage growth stages, the plants were thinned out and only three plants were kept in each pot. MeJA was sprayed at concentrations of under 100 μM [20] at the beginning of the flowering stage. Samples were collected from inflorescence [21]. A two-factorial experiment with two MeJA levels (0 and 100 mM) in four-point times (0, 6, 12, and 24 h) based on a completed block design with three replications was conducted and stored at -80 °C for RNA extraction.

2.2. RNA extraction, cDNA synthesis, and primer designing

Total RNA was extracted from 100 mg of inflorescence using the TRIzol Reagent (Invitrogen, Carlsbad, California, United States), according to the manufacturer's protocol. The RNA quantity and quality were determined with a NanoDrop 2000c Spectrophotometer (Thermo Scientific NanoDrop 2000, USA) and were qualified by 1% agarose gel electrophoresis.

For the miRNA, cDNA was synthesized according to the protocol developed by Varkonyi-Gasic et al (2007) [22] and RT primers (long stem-loop extension primers), according to the instructions of the manufacturers.

The expression level of miR160 and miR166 was determined using the SYBR Green PCR Master Mix. 18s rRNA gene was considered as an internal control. The qRT-PCR was performed in three biological and two technical replicates. The thermal conditions for miRNAs included an initial denaturation step at 95 ^{0}C for 10 minutes, then 32 cycles of 95 ^{0}C for 15 s, 58 to 60 ^{0}C for 30 s and 72 ^{0}C for 30 s. Accordingly, to evaluate miRNAs three types of primers were assigned. These primers are included; SL RT primer which is involved in synthesizing cDNA

from miRNAs (Table 1), forward primers and general primers which are involved in reproducing miRNAs (Table 2), and the target genes primers (Table 3).

Table 1 The primer sequences used in the cDNA synthesis.

MicroRNA name	SL RT primer
miR160	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGGCAT
miR166	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGCGCGACTAGATC

Table 2 The primer sequences of miRNAs used in qRT-PCR validation

MicroRNA name	Forward primer (5' to 3')
miR160	CGATGCCTGGCTCCCTGT
miR166	CACCCACTTTCGACCCTTAAACTCGCTCCA
Universal reverse primer	CCAGTGCAGGGTCCGAGGTA

Table 3 The primer sequences of target genes used in qRT-PCR validation

Gene name	Forward primer (5'to3')	Reverse primer (5'to3')
miR160-ARF17	CGAGGACATCTTCTCTCCACTG	CACGTCTGATGAGTATTCTCGC
miR166-ARF6	CAAAGTTTAGCAGCTACCACGA	ACGTCGTTCTCTCGGTCACGAC

For the potential target genes, the cDNA synthesis was performed according to the instructions of the ProtoScript® First Strand cDNA Synthesis Kit (NEB Company, UK. Cat# E6300S)**.** Expression of target genes was assayed with qRT-PCR according to the protocols mentioned above gene-specific primers for qRT-PCR. The thermal profile was 95°C for 10 min, followed by 35 cycles of 95°C for 10 s and 60°C for 20 s. The SAND gene that was assigned as an internal control gene to prior the the reacher works was affiliated with the medicinal plant of *T. ammi* and is considered a reference gene [22]. Data were analyzed using the comparative cycle threshold (Ct) method. Three technical replicates were used for each biological replicate. PCR reactions were run by using an AB StepOnePlus real-time PCR thermal cycler detection system (Applied Biosystems Applera, Carlsbad, California, USA). The primers were designed using Primer prmier6 software (http://www.premierbiosoft.com/primerdesign/).

Gene Expression Statistical Analysis

Statistical analyses of qRT-PCR results were performed using ANOVA with IBMSPSS 24.0 (SPSS, Inc., Chicago, IL, United States). A significance level of P-value cut-off of < 0.05 was applied.

Physical and genetic (Co-expression) interaction networks were constructed using the GeneMANIA prediction web server, with default parameters (https://genemania.org/) [23]. The expression values of MeJA-responsive miRNAs of *T. ammi* were illustrated as a heatmap. It was plotted by https://www.bioinformatics.com.cn/en, a free online platform for data analysis and visualization.

RESULTS

The results showed that MeJA altered the miRNA response to a challenge. The most pronounced effect of MeJA treatment was a prolonged up- or downregulation of miRNA expression. miRNA expressions showed that miR160 and miR166 were differentially expressed during MeJA treatments at four-point times ($P \leq 0.01$). miR160 reached expression peaks at 6, and 12 h, respectively, but were barely detectable in 24 hrs. This suggests that they have expression-related and functional diversity. In addition, the miR166 which encodes transcription factors, was shown the lowest degree of down-regulation in response to MeJA treatment (Figure. 1). Among the targets, the lowest expression was shown by the miR160 target gene ARF17 in 24hrs. The expression of the miR160 target gene ARF17 showed a significant upregulation following three-point times ($Pvalue \leq 0.01$) (Figure. 2). However, the increase in the miR160 target gene in 24 h was lower than the expression level of 12 h (Figure 1a and b). It should be mentioned here that, plus, the above-mentioned target genes, the evaluated miRNAs can regulate several target genes and be involved in various biological processes.

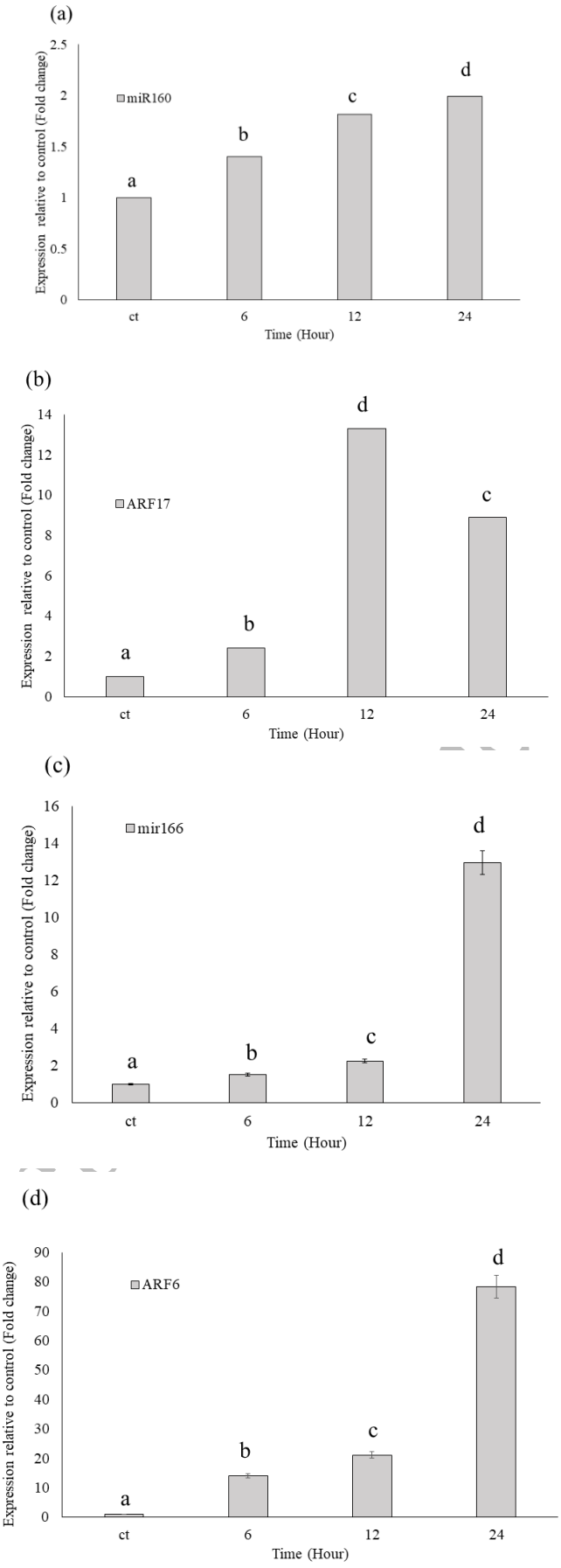

Fig. 1 qRT-PCR expression analysis of miR160 (a), ARF17 (B), miR166 (c) and ARF6 (d) genes following treatment of *Trachyspermum ammi* inflorescence with MeJA (100 mM) at 0, 6, 12, and 24 h post-treatment. Black columns stand for control treatment, and gray columns stand for MeJA treatment. ct: controlled samples. Different lowercase and uppercase letters indicateindicate significant differences in expression between different times, according to the Tukey test ($p \leq 0.05$)

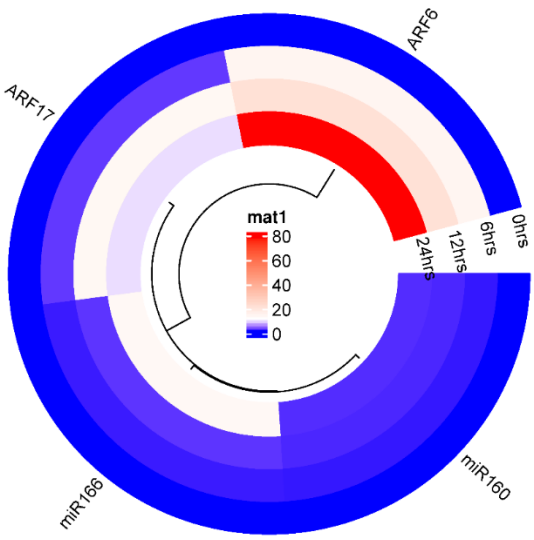

Fig. 2 A Circular heatmap of expression values for the two miRNAs and their targets in response to MeJA treatment. Values were normalized by Z-score normalization. With color changes from blue to red. Blue color indicates lower than mean intensity, and red represents higher than mean intensity. Each row represents a miRNA and each column represents a time ($p < 0.05$, $logFC > |1|$).

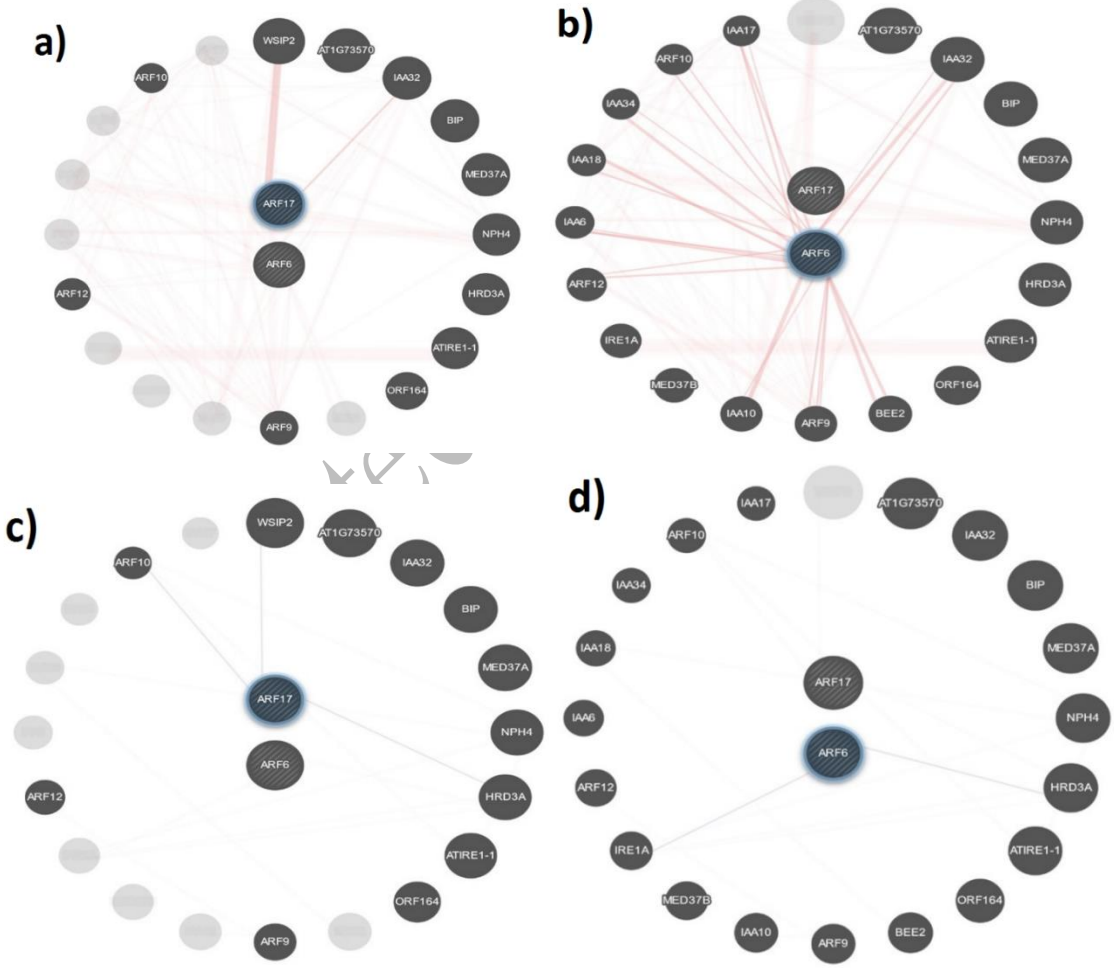

Fig. 3 Physical and genetic network for the target genes (a) genetic network for the ARF17 (b) genetic network for the ARF6 (c) Co-expression network for the ARF17 (d) Co-expression network for the ARF6. Physical network: Protein-protein interaction data. Two gene products are linked if they were found to interact in a protein-protein interaction study. Co-expression network: Two genes are linked if their expression levels are similar across conditions in a gene expression study. Most of these data are collected from the Gene Expression Omnibus (GEO).

To further understand functional metabolites associated with target genes responding to MeJA, co-expressed networks of predicted target genes were separately constructed using the GeneMANIA prediction web server (Figure. 3, Table S1). The result indicates that ARF17 co-expressed together with 3 other proteins: WSIP2 (WUS interaction protein), HRD3A, and ARF10, and on the other hand, ARF6 co-expressed together with ERAD-associated E3 ubiquitin-protein ligase component (HRD3A) and IRE1A. ARF10 and ARF17 are target genes of miR160. miR160 negatively regulates expression of them. WUS-interacting protein (WSIP2) is a member of the transcriptional corepressor-like proteins family and interacts with the WUSCHEL gene (WUS). WUS recruits transcriptional corepressors and thereby suppresses the target genes required for differentiation and thus helps to maintain the meristem [24]. Altogether our results suggest that miR160, possibly through the regulation of ARF10/ARF17 and WSIP2 responds to intrinsic and extrinsic developmental signals. However, further experiments are necessary to determine the interaction network mode.

DISCUSSION

miRNAs are related to the responses of hormones by negatively regulating target genes in hormonal pathways. Many miRNA gene promoters contain hormone response elements, suggesting that miRNA regulation may be a way of hormone response [25]. Several miRNAs that are known to target regulators of the JA pathway were found to be differentially expressed. miR166, which targets the JA biosynthesis regulators ARF6/8, was prolonged downregulated after MeJA treatment. The results of miR160 miR166 expression in response to MeJA at four different times in *T. ammi* showed that miRNAs may be involved in the response of plants to MeJA (Figures 1a and b).

According to previous reports, miR160 is involved in the auxin response by targeting auxin response factors (ARF) genes [26]. Auxin-responsive genes such as ARF10 ،ARF16 and ARF17 act as miR160 target genes which corresponds to previous, studies of miR160-ARF10/16/17 working as a molecular bond [27]. The miR160 family is a highly preserved miRNA that plays a wide spectrum of roles in cellular and physiological processes in the plant. In general, this family consists of several members in plants that might show different functions [11]. The members of miR160 families responded to stimuli such as light, abscisic acid, salicylic acid (SA), and heat stress [28]. The response of pri-miR160 to salicylic acid tended to decrease but, treated with MeJA tended to increase, this approach revealed that pri-miR160 is associated with hormone transmission. The high preserver family of miR160, ARF10, ARF16, and ARF17 negatively regulates and oppresses the ARF family [29]. The families of miR160 and miR166 are highly conserved among plant species [30]. A study on *Arabidopsis* has shown that the effect of miR160 on ARF17 is negatively regulated by the accumulation of MeJA [31], although it was expected similar performance would be observed in another study, such as *Arabidopsis* and tomato plants, however, no relevant changes appeared at the expression level of ARFs that are known targets of miR166, miR319 or miR160 [32]. Moreover, MeJA-treated skin was found to have significant increases in JA levels after injury, but MeJA-treated healthy skin did not show an increase [33]. In *Artemisia annua*, the expression levels of miR160 and miR166 increased with MeJA-treated. Furthermore, the phytohormone-responsive miR160-ARF1 was identified as a negative regulator in *A. annua*. miR167, which targets the JA biosynthesis regulators ARF6/8, was prolonged downregulated after MeJA treatment [34] Hence, it is not clear how miRNAs regulate the biosynthesis of JA in Norway spruce. microRNAs are differentially responded to MeJA [35]. miR166 and miR167 respond to biosynthesis regulation of ARF6 and ARF 8 respectively and then downregulate after MeJA treatment [32]. It also has been documented that miR166 has decreased under the treatment of MeJA in Chinese yew [36]. The results in the Euphorbia plant of genus Forfion showed that microRNAs are involved in the response to MeJA which revealed high expression frequency in miR160 and miR166 families. The reason for general conformity can be that miRNAs in addition to regulating mRNAs, can also control mRNAs translational of targets on the hand other, microRNAs may regulate the expression of mRNAs in response to MeJA. However, further studies are needed to understand this implication in the future.

CONCLUSIONS

The present study included the identification of miRNAs in the *T. ammi* plant via a computational approach by which two miRNAs were assessed to pursue the response of expression levels through MeJA. Furthermore, the validation results showed that several miRNAs, including miR160 and miR166 families in the response to MeJA,

play a significant role in the *T. ammi* plant. However, to better understand how miRNAs regulate the response methyl jasmonate, further analysis of JA pathway biosynthesis and signaling events is required. The findings here suggested that results revealed a theoretical basis for further decryption of miRNA regulatory mechanism in the response to methyl jasmonate and confirmed the performance in target genes in the *T. ammi* plant.

Supplementary Materials

The following supporting information can be downloaded in: Table S1: title; Co-expression network for the target genes.

Author Contributions

Conceptualization, L.N and D.N.; methodology, L.N.; software, S.SH.; validation, L.N., S.SH. and D.N.; formal analysis, S.SH.; investigation, A.P.; data curation, L.N and S.SH; writing—original draft preparation, S.SH.; writing—review and editing, D.N.; visualization, L.N and D.N; supervision, L.N.; project administration, L.N. All authors have read and agreed to the published version of the manuscript.

Consent to Publish

This article does not contain any studies with human or animal subjects.

Conflicts of Interest

The authors declare no conflict of interest.

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