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RESEARCH ARTICLE

Identification of MiR398 and Its Regulatory Roles in Terpenoid Biosynthesis of *Persicaria odorata*

Nursyah Fitri Mahadi, Azman Abd Samad, Abdul Fatah A. Samad*

Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia

Abstract *Persicaria odorata* is an herbaceous plant with antifungal and antibacterial properties. The plant produces secondary metabolites, including phenols, sulphur-containing compounds and terpenoids, in response to biotic and abiotic stresses. Terpenoids in *P. odorata* are synthesized through the mevalonate (MVA) and the methylerythritol phosphate (MEP) pathways. 1-deoxy-D-xylulose-5phosphate isomerase (DXR) is a rate-limiting enzyme in the MEP pathway and may be regulated by microRNA (miRNA) miR398. There is a lack of evidence showing miR398 regulation in terpenoid biosynthesis in *P. odorata* through DXR-targeting. The study aimed to verify the stem-loop structure of miR398 and analyse its expression towards the target gene, DXR, in treated and control P. odorata. RNA was quantified and qualitatively analysed using a Nanodrop spectrophotometer and agarose gel electrophoresis. The stem loop of miR398 was verified using reverse transcriptase PCR (RT-PCR) and agarose gel electrophoresis. The expression of miR398 and DXR was compared between control and treated samples. Treated leaves were punctured with needles and left for 48h before harvest. Gene expressions were quantified and normalised using reference genes. The stem-loop structure of miR398 was confirmed, despite possible primer mismatches and unspecific binding. This step was essential before comparing and assessing the gene expression of miR398 and DXR. A decrease in abundance of miR398 whereas in increase in abundance was in the treated sample compared to the control sample indicating that miR398 negatively regulated DXR under stress conditions, suggesting an increase in terpenoid synthesis as a defence mechanism. DXR acts as a rate-limiting enzyme in the MEP metabolic pathway. Further studies are needed to quantify the effects of miR398 on terpenoid biosynthesis after wounding in *P. odorata*.

Keywords: MicroRNA, *Persicaria odorata*, plant stress, terpenoid biosynthesis, gene expression regulation.

***For correspondence:**
abdulfatah@utm.my

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Introduction

Persicaria odorata or commonly known as Vietnamese coriander and *kesum*, is an edible herb that is widely planted in Southeast Asian countries, such as Vietnam, Cambodia, and Peninsular Malaysia. The leaves have been utilised as additive in Southeast Asian cuisines and traditional medicines [1]. However, stresses can interfere with the plant growth by causing physiological responses and biological damages [2,3]. Abiotic stresses such as high and low temperature, frequent precipitation, drought, and salinity, can impact the plant growth significantly, leading to lower yield. Meanwhile, biotic stress affects the plant via herbivorous or pathogenic attack, where these stresses should be highlighted in efforts mitigate the impairment caused by the infection [2].

Furthermore, previous studies have shown that the aromatic and medicinal application of *P. odorata* leaves can be contributed to the various chemical constituents, including terpenoids [1]. Phytochemical analysis revealed the presence of oxidised monoterpenes, diterpenes, and sesquiterpenes in the aerial parts of the plant, as well as triterpenoids from the leaves [4-6]. In plants, secondary metabolites (SMs)

are classified into three categories, including phenolic compounds, nitrogen/sulphur-containing compounds, and terpenoids. The fundamental building block of terpenoids is the toxic 5-C isoterpenoid, which discourages invasion by herbivores [7].

Terpenoids are commonly found in herbs such as *P. odorata* and synthesised through the mevalonate pathway (MVA) and methyl erythritol pathway (MEP) [8]. In specific, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) is the rate limiting enzyme of MEP, where it is responsible to catalyse the rearrangement of 1-deoxy-D-xylulose-5-phosphate (DXP) into 2-C-methyl-D-erythrose 4-phosphate [8,9]. These secondary metabolites contribute to the antimicrobial and antioxidant properties of *P. odorata*, as shown in previous studies [4,6,10].

One of the plant's responses to biotic and abiotic stress is production of secondary metabolites. To regulate this response, microRNA (miRNAs) can be employed, as post-translational regulation approach. miRNAs are short, non-coding regulatory RNAs that are transcribed from genomic DNA by RNA polymerase II. It has a stem loop structure and is expressed first into primary miRNA (pri-miRNA), before converted into mature miRNA. MiRNA regulatory effects are inhibitory, either by the cleavage of target mRNA or by inhibiting the translation of the target mRNA [11]. Several studies have demonstrated the regulation of SMs biosynthesis, including terpenoids during stress using miRNA [12-14].

In brief, as biotic and abiotic stress strikes the plant, terpenoids are synthesised via these pathways. In response to this, miRNA is expressed to modulate the production of terpenoid, where the target is gene specific. A recent study has shown the role of miR398 towards the terpenoid biosynthetic pathway by the regulation of DXR [14]. Despite, there is a lack of information regarding miR398 in *P. odorata*. In addition, the application of wounding stress on the plant may influence the miR398 and its target gene, which may subsequently affect the terpenoid biosynthetic pathway. Hence, this study was employed to detect the presence of miR398 through the identification of its stem-loop structure through reverse transcriptase PCR (RT-PCR). The regulatory role of miR398 towards the target gene and terpenoid biosynthesis in the plant was also explored through gene expression analysis.

Materials and Methods

The leaf samples for the control and treated (wounded) *P. odorata* were sourced from Agrotani, Universiti Teknologi Malaysia, Skudai Johor, Malaysia. The control and treated plants were labelled accordingly before the lateral leaves of the treated plant were punctured with needles on each leaf. Four incisions were made on each leaf of the treated plant. The plants were left in the same environment for 48 hours before the leaves were harvested. During harvest, the leaves were quickly moved into frozen 50 mL RNase-free Falcon tubes, then submerged into liquid nitrogen in a portable tank [15]. The leaves samples were stored at -80^{o}C.

RNA Extraction

RNA extraction preparation was made by chilling the equipment required for the extraction process. The mortar and pestle, spatula, forceps, and RNase-free 2.5 mL microcentrifuge tubes were chilled using liquid nitrogen. The frozen leaves were prepared for extraction by grinding it using the chilled mortar and pestle into very fine powder, and occasionally liquid nitrogen was added to prevent thawing of the samples [16]. After the fine, frozen powder of each sample was transferred into its respective microcentrifuge tubes, the total RNAs of the samples were extracted using cold PureLink® Plant RNA Reagent (Thermo Fisher Scientific) by following the provided protocol. At the last step, 20 µL of nuclease-free water was added into the RNA pellet. The homogenised solution of the total RNA of each sample was then stored immediately at -80^{o}C to prevent RNA degradation.

Quality control measures was carried out to ensure the adequate quantity and quality of the total RNA samples. Nanodrop spectrophotometer (ND-1000) assessed the purity of the RNA samples and ratio of absorbance at 260 nm and 280nm. A "pure" RNA sample has ratio approximately 2.0. In addition, the 260/230 ratio was also employed as a secondary measure for the purity of RNA samples and presence of contaminants [17,18]. Subsequently, the quality or integrity of the RNA samples was verified through agarose gel electrophoresis. First, 1.0% agarose solution was prepared by adding 1.5 g of Certified Molecular Biology Agarose (Bio-Rad) in 100 ml of 1X TAE buffer, pH 8.5 (Thermo Fisher Scientific) in a RNase-free conical flask. The agarose solution was microwaved and after the agarose solution was slightly cooled down, 4 µL of the GreenSafe DNA Gel Stain (Canvax Biotech) was added into each 50 mL of the solution. Lastly, the solution was poured into the gel case to be solidified.

The solidified gel was then submerged in 1X TAE buffer. ExactMark 100bp DNA ladder (100-1500 bp) (1st Base) and RNA samples were prepared. For the DNA ladder, 5 µL of ladder solution and 1 µL of nuclease-free water was mixed and resuspended with micropipette. Then, a total of 6 µL RNA ladder solution was pipetted into the first well cautiously, to avoid puncturing the gel. As for the RNA samples, a mixture of 1 µL of loading dye, 2 µL of nuclease-free water, 3 µL RNA sample was prepared and pipetted into the wells. The agarose gel electrophoresis ran for 60 minutes with constant voltage of 90 V. Next, the gel image was captured under UV transillumination [19].

Primer Design and cDNA Synthesis

Before proceeding with polymerase chain reaction (PCR), suitable reverse and forward primers were designed for the target DNA, i.e., DXR gene, and miR398. Primer3 tool was utilised to design primers for miR398 and DXR gene as shown in Table 1.

Table 1. Primers sequences for target gene, reference genes, and miRNA

Gene/miRNA	Primer sequence (5' to 3')	Melting temperature, T_m (^oC)
DXR	Forward: TAA AGC CCC AGA CAA CGT GA	56.4
	Reverse: GGT CAG CTC AAC AAC CTT GA	55.2
ata-miR398g	Forward: TGG GGT CGA GCT GGG AAC	60.0
	Reverse: ACA CAT GAG TTG ATA AGA GAC GC	54.9
Tubulin	Forward: TAC CAG CCA CCA ACC GTA GTC C	56.7
	Reverse: CCA ACC TCC TCG TAG TCT TTC TCA A	56.3
tRNA	Forward: GCT TCA GGG ATT GAG CTT TG	60.9
	Reverse: GGA ATT CCA TCC ACC ACA TC	58.0

As for the mir398, the stem-loop sequence of miR398 conserved to *Aegilops tauschii* was searched and browsed in miRbase database. The loop sequence of the ata-miR398g with accession name, MI0031718 was identified (5'-GAT TGA C-3'). Next, the stem loop sequence was queried as input in Primer3. The most suitable primer pairs were selected according to the melting temperature, T_m, GC content, complementarity, and hairpin formation, before the sequence was sent to Integrated DNA Technologies (IDT), Singapore for primers assembly. Furthermore, the primer sequence for each of the reference genes, tubulin and tRNA was also identified using the same method.

After the primers were received, each primer was diluted accordingly. Primer stock with concentration of 100 µM was prepared by adding DNase free water into the tube with dry primer, according to the amount stated on the manufacturer sheet. Then, each of the primer stock was mixed and centrifuged briefly. Next, working stock for each primer were prepared by dispensing 10 µL from the primer stock into a new centrifuge tube and 90 µL of DNase free water was added afterwards. The working stocks were then mixed and centrifuged briefly. The primers were then diluted according to the manufacturer, and the stock solution for each primer was prepared and stored at -20^{o}C.

For cDNA synthesis, total RNA samples for each control and treated sample were thawed, mixed and centrifuged briefly. The cDNA for each sample was synthesised using the RevertAid First Strand cDNA Synthesis Kit, according to the steps given by manufacturer (Thermo Fisher Scientific). The synthesised cDNAs were then stored at -70^{o}C.

Reverse Transcriptase PCR (RT-PCR)

All RT-PCR in this experiment were carried out according to the One*Taq*® Quick-Load® 2X Master Mix with Standard Buffer (New England Biolabs).

First, RT-PCR was implemented to verify the presence of miR398's stem loop structure. The specific primer, as stated in Table 1, with its respective annealing temperature, T_a (48^{o}C) to detect miR398g, was utilised. Meanwhile, 3% (w/v) agarose gel was prepared for the gel electrophoresis with pH of 8.5 buffer was mixed and microwaved until dissolved and clear. 4 uL of GreenSafe DNA Gel Stain (Canvax Biotech) was pipetted into the 50 mL agarose solution. For gel electrophoresis, 1 uL of 6X TriTrack DNA Loading Dye (Thermo Fisher Scientific) was used as the loading dye. 5 uL GeneRuler Ultra Low Range (ULR) DNA Ladder (Thermo Fisher Scientific) was pipetted into the first well as ladder. Then, 10 uL of the miR398 PCR product of the control sample was pipetted into the next well. Electrophoresis of the gel was carried out for 60 minutes at constant voltage of 90 V. The gel was then viewed with a Gel Documentation System (Bio-Rad).

The next RT-PCR was carried out for miR398 and DXR gene expression analysis. The same master mix and protocol was followed for this reaction. For DXR expression analysis, four PCR tubes were prepared, which included control and treated PCR for DXR, and control and treated PCR for tubulin as the reference gene. Meanwhile, miR398 expression analysis also involved four PCR tubes with control and treated PCR of miR398, and control and treated PCR for tRNA as the reference gene. PCR was conducted using same thermocycling conditions as the first PCR, with different T_a for DXR (53ºC), tubulin (55ºC), and tRNA (51ºC). The amplicons for the respective expression analysis samples were ran on gel electrophoresis with 3% (w/v) agarose gel for 60 minutes, at 90 V constant voltage. A negative control was also included for each gel. The gels were documented digitally using Gel Documentation System (Bio-Rad).

Gene Expression Analysis

The expression gel of DXR and miR398 of treated and control plants was analysed using Image J software (National Institute of Health, USA). The gel images of the target gene and miR398 of both treated and control sample were compared visually using the software. The results in the form of area of the peak (%) obtained were interpreted as expression values. Normalisation of the DXR or miR398 bands were completed by comparing each band with its respective reference gene's band. The relative expression for DXR and miR398 was calculated by dividing the percentage of target gene or miRNA with the percentage of their respective reference gene. The difference of relative expression value between the target gene and miRNA of control and treated samples were acquired.

Results and Discussion

MiRNA is utilised in the cell as a post transcriptional regulatory tools in the cells. Thus, in order to elucidate the effect of miRNA, specifically miR398 towards terpenoids biosynthesis in *P. odorata*, total RNA from the samples were extracted carefully and miR398 in the total RNA was detected using specific primer that binds to the stem-loop sequence of the miRNA. Moreover, the amplicons were analysed through its gene expression visualised on 3% (w/v) agarose gel and compared using Image J.

Total RNA Quantification and Quality Assessment

The extracted RNA samples were validated on its total RNA quantity or yield and purity through Nanodrop spectrophotometer. The concentration of RNA in the sample is determined by measuring absorbance at 260 nm. The purity of total RNA is determined by comparing the absorbance at 260 nm (A_{260}) with absorbance at 280 nm (A_{280}) and 230 nm (A_{230}) [20,21]. Based on Table 2, the yield of the control and treated samples were high, with concentration of 1420.0 ng/µL and 2208.9 ng/µL, respectively. Meanwhile, the A_{260}/A_{280} ratio was 2.11 and 2.13 for control and treated sample, respectively. The A_{260}/A_{230} ratio for control sample was 1.81 and 1.98 for treated sample.

Table 2. The purity and yield of total RNA from control and treated *P. odorata* samples

P. odorata sample	Yield (ng/µL)	A_{260}/A_{280} ratio	A_{260}/A_{230} ratio
Control	1420.0	2.11	1.81
Treated (Wounded)	2208.9	2.13	1.98

The results shown high yields of total RNA from both sample, which can be owed to the efficiency of extraction method and reduced delay time for sample calibration. Total RNA from *P. odorata* leaves samples were extracted using commercial kit method, i.e., PureLink® Plant RNA Reagent (Thermo Fisher). The total RNA extracted through this method has resulted to high yield of total RNA. Moreover, previous studies that utilised tis kit for RNA extraction have also shown high RNA yield from leaves of other dicotyledons such as *Nicotiana benthamiana* (1946.0 ng/µL), *Bursera* sp. (601.0 ng/µL), *Biophytum sensitivum* (2289.7 ng/µL), and *Centella asiatica* (1664.3 ng/µL) [22,23].

The purity ratios of the total RNA varied between samples based on the results, the A_{260}/A_{280} ratio for the control and treated samples were close to the recommended value of 2.0, i.e., 2.11 and 2.13, respectively. Typically, the accepted value of the A_{260}/A_{280} ratio for "pure" RNA samples is around 2.0 to 2.1. A lower ratio suggests the presence of contaminants such as protein and phenolic compounds, which absorb strongly at or near 280nm [17,24,25]. Hence why the samples can be accepted as sample with high purity of RNA. These A_{260}/A_{280} values are evident to the accepted values in other plants studied such as *Eucalyptus grandis* (2.17), wheat (1.98), and *Centella asiatica* (2.02) [23,26,27].

Furthermore, A_{260}/A_{230} ratio was used as a secondary measure in assessing the RNA purity. The results presented the samples with values of 1.81 and 1.98 A_{260}/A_{230} for control and treated RNA samples, respectively. The "pure" nucleic acid A_{260}/A_{230} readings are usually greater than the corresponding A_{260}/A_{280} values, in the range of 2.0-2.3. The value determines the presence and amount of contaminants that absorbs strongly at 230 nm such as guanidine thiocyanate and phenol, commonly used in RNA extraction and purification [25]. The slightly reduced A_{260}/A_{230} suggests interference, which may be caused by contamination with polysaccharides and secondary metabolites such as polyphenols from the plant, in addition to guanidine thiocyanate and phenol, as the RNA samples were extracted with plants [23]. Similar A_{260}/A_{230} ratios were accepted in *Cuminum cyminum* with A_{260}/A_{230} ratio of 1.94 when extracted using Plant RNeasy kit (Qiagen) [28]. Therefore, the total RNA extracted from *P. odorata* control and treated samples have shown high yield and good absorbance ratio. This highlights the importance of quantification and purity assessment of the RNA samples for molecular analyses, including PCR amplification and gene expression analysis.

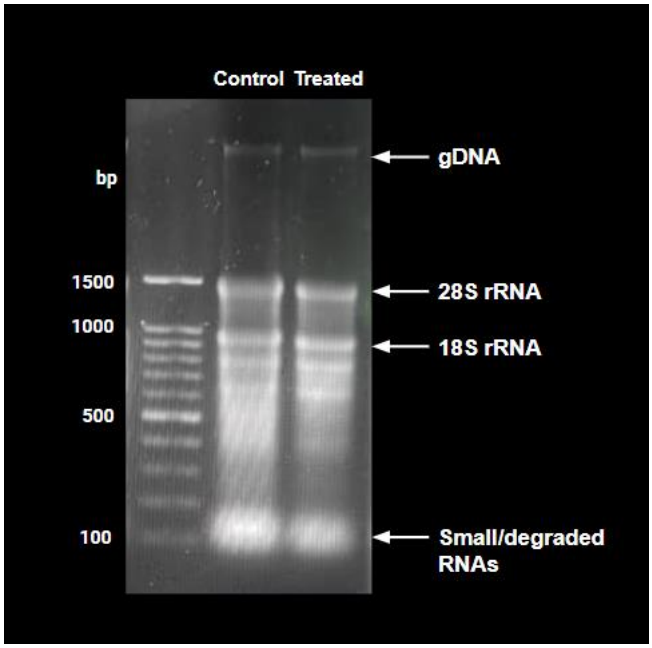

Figure 1. Quality assessment of total RNA samples from control and treated sample of *P. odorata* on 1% (w/v) agarose gel

To further assess the quality of the total RNA samples, RNA integrity gel using 1% (w/v) agarose were conducted. Based on Figure 1, the two bands were observed around 1500 bp and 1000 bp in each sample. Furthermore, the bands below 1000 bp shown smearing for both samples and several other bands were separated. There were also bands at lower size of approximately 100 bp in each lane. In addition, bands at the size more than 1500 bp were detected in the gel for both samples. RNA quality refers to its integrity, where ribosomal RNA (rRNA) has been utilised as quality indicator [29]. In RNA quality assessment, the distinct appearance of 28S and 18S rRNA bands in the gel suggested that the RNA samples were in good quality, as shown in Figure 1. Nonetheless, the smearing of both bands reduced the integrity of the RNA, implying that the RNA in both samples were partially degraded [19].

Despite, the partial fragmentation of RNA did not affect the amplification products of the samples, especially if the fragments to be analysed is within 300 to 700 nucleotides. This is based on the rationale that the flanked regions of the RNA by specific primers still contributed to the amplification product, in spite of fragmentation and degradation of total RNA [30]. Additionally, certain degradation of RNA in samples is inevitable, due to internal regulatory processes, especially the cell's mechanism in preventing RNA accumulation in the cells. This lead to the inevitable degradation of some RNA by the endonucleases produced in the cells [31]. Hence, prevention measures can be taken to minimise the effect of endonucleases towards RNA in the samples. The measures included the treatment of apparatus and materials with 0.1% DEPC, which has been carried out throughout this experiment. Therefore, partially degraded control and treated samples can still be applied for PCR purposes where it involves the amplification of the small amount of miRNA in the samples.

MiR398 Stem Loop Verification

Reverse transcriptase PCR was implemented to amplify the miR398 in both samples, after cDNA was synthesised from the RNA samples. The amplicons were visualized on the 3% (w/v) agarose gel, where the products appeared as bands. Figure 2 shows the 3% (w/v) agarose gel electrophoresis of the miR398 amplicon of the control sample. A faint band can be observed at the size of approximately 250 bp.

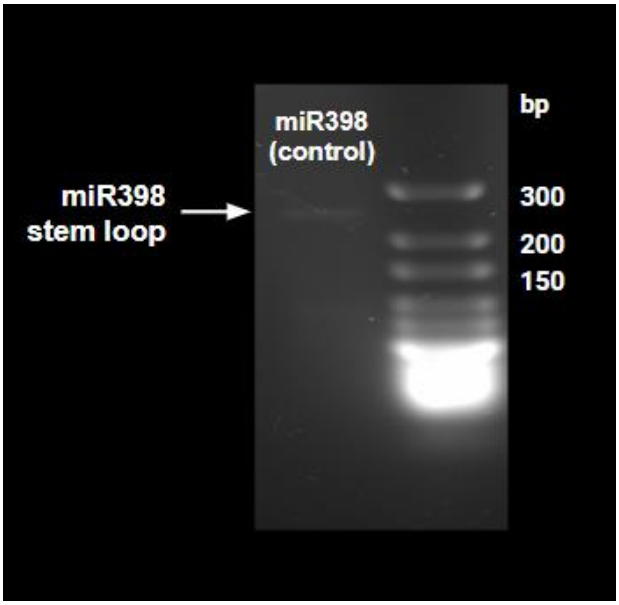

Figure 2. MiR398 stem loop verification on 3% (w/v) agarose gel

The band suggested the presence of the stem loop structure of the miRNA. This is possible due to the use of specific primers to amplify the miRNA, where the primers were designed to include the stem loop sequence of its target, i.e., miR398 [32,33]. This implies that the isomer of miRNA detected was the precursor miRNA (pre-miRNA) of miR398. Pre-miRNA is derived from primary miRNA (pri-miRNA), resulting in a stem-loop structure of the pre-miRNA. Subsequently, the pre-miRNA is processed into short double-stranded miRNA by Dicer, before forming a single-stranded mature miRNA [32,34]. Furthermore, the band appeared to be faint, which indicates low level of miR398 amplified in the control sample. The availability of endogenous miRNA such as miR398 after its biogenesis is universally low and its concentration in P. odorata is so far unknown [35]. This poses a challenge in miRNA detection, especially when semi-quantitative method, i.e., reverse transcriptase PCR is utilised, such in this case [36].

Nonetheless, this method was proved dependable, as the primer designed to amplify miR398 is specific to amplify the stem-loop region of the miRNA. This marked the importance of proper primer design and primers testing and optimisation of PCR parameters, including melting temperatures, T_m of the primer pairs [37]. The observable band indicated that the primer is working, although more optimisation steps may be required to increase the intensity of the band. The steps can include annealing and extension time, as well as template quantity and quality [38]. Moreover, the shorter primer length for miRNA amplification is speculated to cause primer mismatching to the regions within the cDNA of miRNA, which leads to the different amplicon size, 250 bp compared to the expected product size of 70 bp. Despite, the appearance of band on the gel reflects satisfactory amplification of miR398's cDNA. Hence, with the verification of the miR398 stem loop, the control and treated samples were subsequently subjected to gene expression analysis.

Gene Expression Analysis of miR398 and its Target Gene, DXR

The control and treated samples of *P. odorata* were amplified using primer pairs specific to the gene. The amplicons were then run on 3% (w/v) agarose gel electrophoresis and bands of varying intensity were observed, as shown in Figure 3.

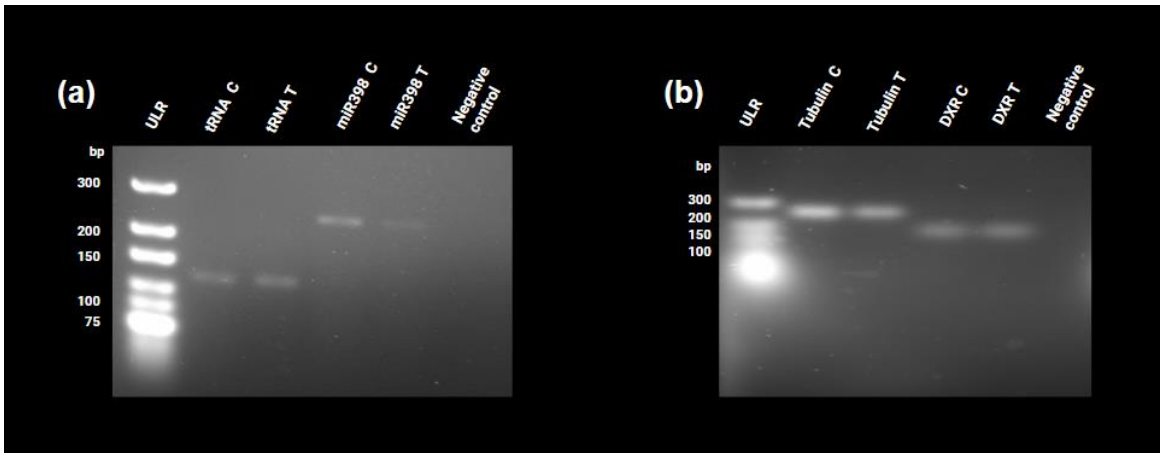

Figure 3. Expression of (a) miR398 and (b) DXR visualised on 3% (w/v) agarose gel. ULR, ultra-low ladder; tRNA C, tRNA control; tRNA T, tRNA treated; miR398 C, miR398 control; miR398 T, miR398 treated; Tubulin C, Tubulin control; Tubulin T, Tubulin treated; DXR C, DXR control; DXR T; DXR treated

Bands for cDNAs of tRNA, miR398, tubulin, and DXR in control and treated samples appeared without smearing and dimers. The product size for tRNA and tubulin was approximately 125 bp and 250 bp. In addition, the absence of smears and dimers suggested the amplification of the reference genes specific to the primer pairs applied. Since the primers are specific to the cDNAs of tRNA and tubulin, it proved the bands appeared were the product of tRNA and tubulin's PCR amplification. The area of the peak for tRNA and tubulin amplicon, which were visualised by the bands' intensity, were similar between control and treated sample. This observation indicated that the expression of these genes in the plant were stable and invulnerable to environmental stresses [39]. A stably expressed reference gene is vital in the aspect of data normalisation, for good accuracy of the target genes' expression level. It is important to minimise the variability due to sample preparation, RNA quantity, and PCR efficiency [40]. Hence, reference gene shown to aid minimising variations by normalising the target gene's mRNA level or target miRNA level with the reference gene's mRNA level after reverse transcription the sequences into cDNA [29,41,42].

The band intensity of miR398 and DXR amplicons were quantified using ImageJ software. The area of the peak of each band represents the signal within the region of interest, i.e., the band selected in the rectangle box. The normalisation of the miR398 and DXR bands were carried out by dividing the percentage of target gene with that of the reference gene, to obtain the relative expression of the gene of interest. The relative expression of each band was as tabulated in Table 3. Relative expression of miR398 in control and treated sample of *P. odorata* was 3.28 and 0.72, respectively. The treated sample had higher expression of miR398 than that of control sample. In other words, miR398 was downregulated after the application of wounding onto the plant. Meanwhile, DXR has relative expression value of 0.94 in control sample and 1.09 in treated sample. An increase in expression of DXR was observed in the treated sample, which suggests the upregulation of DXR post-wounding. The regulatory effect of miR398 towards DXR can also be examined through the expression analysis. Based on the results obtained, miR398 expression was downregulated after wounding, the expression level of DXR was upregulated.

Table 3. Relative expression of miR398 and DXR in control and treated samples of *P. odorata*

Sample	Control	Treated
MiR398	3.28	0.72
DXR	0.94	1.09

Terpenoid biosynthesis in plants including *P. odorata* comprised of the cytosolic MVA pathway and plastidial MEP pathway. DXR, as one of the rate-limiting enzymes involved in the MEP pathway, functions to catalyse the intramolecular arrangement of DXP into MEP in the second step of the pathway [8]. The role of DXR as the rate-determining enzyme of MEP pathway in the production of terpenoids

and its precursors is evident based on previous studies. In the roots of *Salvia sclarea*, the cyanobacterial and orthologous plant DXR gene were transformed into the plant's hairy roots and subsequently, overexpressed. The overexpression of DXR in transformants shown evident increase in diterpenes production, which suggested the importance of DXR in the upregulating MEP pathway [43]. Additionally, overexpression of DXR in *Elizabethkingia meningoseptica* contributed in a synergistic effect that caused the increase in menaquinone production [44]. Thus, the designation of DXR as the gene of interest in this study is relevant to examine the regulatory effect of miRNA on MEP pathway.

On the other hand, miRNA is expressed in the cell, and it has been shown that environmental changes can modulate its expression. In the cell, miRNA act as a regulatory tool, where it can exert it function by inhibiting the translation of the target gene's mRNA. This inhibition can occur by translational inhibition or mRNA degradation, depending to the nature and degree of base pairing between the miRNA and its target gene. MiR398 as post-transcriptional regulatory tool has various targets in pathways within the plant under different types of stress. Previous study on target prediction and relative expression revealed DXR as miR398 target gene in *P. minor* [14]. MiR398 upregulation led to the downregulation of DXR expression under wounding treatment in *P. odarata*, that mimics the biotic and abiotic stress in the environment caused by visible injury to the leaves. In contrast, the expression analysis of miR398 and DXR in *P. minor* showed upregulation of miR398 when induced by abiotic stress, i.e., *Fusarium oxysporum* infection, causing the downregulation of DXR gene [14].

Nonetheless, miR398 have shown to be downregulated when exposed to abiotic and/or biotic stress in other studies. Expression of miR398 in *Triticum aestivum* L. was downregulated under biotic stress, i.e., infection by *Fusarium culmorum* and *Trichoderma* spp. [45]. Correspondingly, copper stress in grapevine modulated the correlation between miR398 and its target genes, the Copper/Zinc super dismutase genes, *CSD1* and *CSD2* negatively [46]. Similar observation was also made between miR398 and *CSD* genes in *Arabidopsis thaliana* [47]. These plants that were affected by copper stress exhibited a decrease in reactive oxygen species (ROS) activities due to the higher production of superoxide dismutase (SOD) accumulation, which counteracts the ROS [45,47]. The examination of the physiological indicators of the stresses visualised the outcome of the miR398 regulatory effects on these plants. Therefore, experimental confirmation on the effect of miR398 downregulation towards terpenoid biosynthesis can be carried out to further validates the regulatory role of this miRNA in *P. odorata* specifically.

These findings implied that modulation by miR398 expression prompted a regulatory loop onto the target genes, including DXR, that is critical in the manipulation of stress tolerance in plants, in this case, *P. odorata*. Therefore, by comparing the miR398 response towards stresses and its effect on the target gene, DXR in this experiment and other studies, indications that the regulatory pattern of miR398 depends on the target gene, plant species, and stresses can be construed. Furthermore, the degree of miR398 response through its expression speculated that miR398 is stress-sensitive, which is essential as a medium to elucidate the effects of stresses toward the biosynthetic pathways where the target gene is involved.

Conclusions

In conclusion, the results from the RNA quantification and integrity assessment revealed the presence of good quality and purity RNA in the total RNA of control and treated *P. odorata* samples, based on its yield and purity ratios of A_{260}/A_{280} and A_{260}/A_{230}. Additionally, the total RNA samples have implied that the RNA was indeed intact enough to be utilised for application in PCR amplification. The verification of the miR398's stem loop structure was carried out and stem loop structure was detected and verified through specific primers that amplified the stem loop region and PCR amplification of cDNA. Hence, following the outcome of the stem loop verification, expression analysis of the miR398 and its target gene in *P. odorata*, DXR was executed. The outcome from the gene expression visualisation through gel electrophoresis bands were quantified using ImageJ. The results suggest correlation between miR398 and DXR expression under wounding treatment. To be exact, miR398 expression was downregulated, while DXR expression was upregulated, from the experiment. Therefore, miR398 is implied to negatively regulates DXR of the MEP pathway, where the cascading effect may cause the increase in production of terpenoids in the plant cells as defence mechanism towards the stresses.

Future works are necessary to further elucidate the effects of modulating and manipulating the regulatory function of miR398 towards DXR under various stress conditions. Moreover, the identification of non-conserved miR398 target genes in various plant species by sequencing is another point of focus in future research, and more functional work is necessary to elucidate the mechanism of action of these species-specific targets [48].

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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References

[1] Shavandi, M. A., Haddadian, Z., Ismail, M. H. S. (2012). *Eryngium foetidum* L., *Coriandrum sativum* and *Persicaria odorata* L.: A review. *Journal of Aisan Scientific Research*, *2*, 410-426. Doi: http://aessweb.com/journal-detail.php?id=5003.

[2] Rejeb, I., Pastor, V., Mauch-Mani, B. (2014). Plant responses to simultaneous biotic and abiotic stress: molecular mechanisms. *Plants*, *3*, 458-475. Doi:10.3390/plants3040458.

[3] Dresselhaus, T., Hückelhoven, R. (2018). Biotic and Abiotic stress responses in crop plants. *Agronomy*, *8*, 267. Doi:10.3390/agronomy8110267.

[4] 4Nguen, V. T., Nguyen, M. T., Nguyen, N. Q., Truc, T. T. (2020). Phytochemical screening, antioxidant activities, total phenolics and flavonoids content of leaves from *Persicaria odorata* Polygonaceae. *IOP Conf Ser-Mat Sci.* 991. Doi:10.1088/1757-899x/991/1/012029.

[5] Yanpirat, P., Vajrodaya, S. (2015). Antifungal activity of *Persicaria odorata* extract against anthracnose caused by *Colletotrcihum capsici* and *Colletotrichum gloeosporioides*. *Malaysian Applied Biology*, *44*, 69-73.

[6] Řebíčková, K., Bajer, T., Šilha, D., Houdková, M., Ventura, K., Bajerová, P. (2020). Chemical composition and determination of the antibacterial activity of essential oils in liquid and vapor phases extracted from two different Southeast Asian Herbs—*Houttuynia cordata* (Saururaceae) and *Persicaria odorata* (Polygonaceae). *Molecules. 25*, 2432. Doi:10.3390/molecules25102432.

[7] Zaynab, M., Fatima, M., Abbas, S., Sharif, Y., Umair, M., Zafar, M. H., Bahadar, K. (2018). Role of secondary metabolites in plant defense against pathogens. *Microbial Pathogenesis*, *124*, 198-202. Doi: https://doi.org/10.1016/j.micpath.2018.08.034.

[8] Tholl, D. (2015). Biosynthesis and biological functions of terpenoids in plants. *Adv Biochem Eng Biotechnol*, *148*, 63-106. Doi:10.1007/10_2014_295.

[9] Pütter, K. M., Van Deenen, N., Unland, K., Prüfer, D., Schulze Gronover, C. (2017). Isoprenoid biosynthesis in dandelion latex is enhanced by the overexpression of three key enzymes involved in the mevalonate pathway. *BMC Plant Biology. 17*. Doi:10.1186/s12870-017-1036-0.

[10] Ridzuan, P. M., Hairul Aini, H., Norazian, M. H., Shah, A., Roesnita, Aminah, K. S. (2013). Antibacterial and Antifungal Properties of *Persicaria odorata* Leaf Against Pathogenic Bacteria and Fungi. *The Open Conference Proceedings Journal*, *4*, 71-74. Doi:http://dx.doi.org/10.2174/2210289201304020071.

[11] Grabowska, A., Bhat, S. S., Smoczynska, A., Bielewicz, D., Jarmolowski, A., Kulinska, Z. S. (2020). Regulation of plant microRNA biogenesis. *Plant microRNAs, Shaping Development and Environmental Responses*, Miguel, C., Dalmay, T., Chaves, I., Eds. Springer Nature. *1*, 3-24.

[12] Sobhani Najafabadi, A., Naghavi, M. R. (2018). Mining *Ferula gummosa* transcriptome to identify miRNAs involved in the regulation and biosynthesis of terpenes. *Gene*, *645*, 41-47. Doi: https://doi.org/10.1016/j.gene.2017.12.035.

[13] Zhao, S., Wang, X., Yan, X., Guo, L., Mi, X., Xu, Q., Zhu, J., Wu, A., Liu, L., Wei, C. (2018)**.** Revealing of MicroRNA involved regulatory gene networks on terpenoid biosynthesis in *Camellia sinensis* in different growing time points. *Journal of Agricultural and Food Chemistry*, *66*, 12604-12616. Doi:10.1021/acs.jafc.8b05345.

[14] Samad, A. F. A., Rahnamaie-Tajadod, R., Sajad, M., Jani, J., Murad, A. M. A., Noor, N. M., Ismail, I. (2019). Regulation of terpenoid biosynthesis by miRNA in Persicaria minor induced by Fusarium oxysporum. *BMC Genomics*, *20*, 586. Doi:10.1186/s12864-019-5954-0.

[15] Tang, S., Wang, Y., Li, Z., Gui, Y., Xiao, B., Xie, J., Zhu, Q.-H., Fan, L. (2012). Identification of wounding and topping responsive small RNAs in tobacco (*Nicotiana tabacum*). *BMC Plant Biology. 12*, 28. Doi:10.1186/1471-2229-12-28.

[16] Gismondi, A., Di Marco, G., Canini, A. (2017). Detection of plant microRNAs in honey. *PLoS One*, *12*, e0172981. Doi:10.1371/journal.pone.0172981.

[17] Garcia-Alegria, A. M., Anduro-Corona, I., Perez-Martinez, C. J., Guadalupe Corella-Madueno, M. A., Rascon-Duran, M. L., Astiazaran-Garcia, H. (2020). Quantification of DNA through the NanoDrop spectrophotometer: methodological validation using standard reference material and Sprague Dawley Rat and Hhuman DNA. *Int J Anal Chem., 2020*, 8896738. Doi:10.1155/2020/8896738.

[18] Passos, M. L., Saraiva, M. F. S., Lúcia, M. (2019). Detection in UV-visible spectrophotometry: Detectors, detection systems, and detection strategies. *Measurement, 135*, 896-904. Doi:10.1016/j.measurement.2018.12.045.

[19] Aranda, P. S., LaJoie, D. M., Jorcyk, C. L. (2012). Bleach gel: A simple agarose gel for analyzing RNA quality. *Electrophoresis, 33*, 366-369. Doi:10.1002/elps.201100335.

[20] Garcia-Elias, A., Alloza, L., Puigdecanet, E., Nonell, L., Tajes, M., Curado, J., Enjuanes, C., Diaz, O.,

Bruguera, J., Marti-Almor, J., *et al*. (2017). Defining quantification methods and optimizing protocols for microarray hybridization of circulating microRNAs. *Sci Rep*, *7*, 7725. Doi:10.1038/s41598-017-08134-3.
[21] Masago, K., Fujita, S., Oya, Y., Takahashi, Y., Matsushita, H., Sasaki, E., Kuroda, H. (2021). Comparison between fluorimetry (Qubit) and spectrophotometry (NanoDrop) in the quantification of DNA and RNA extracted from frozen and FFPE tissues from lung cancer patients: A real-world use of genomic tests. *Medicina. 57*, 1375. Doi:10.3390/medicina57121375.
[22] Yockteng, R., Almeida, A. M. R., Yee, S., Andre, T., Hill, C., Specht, C. D. (2013). A method for extracting high-quality RNA from diverse plants for next-generation sequencing and gene expression analyses. *Applications in Plant Sciences. 1*, 1300070. Doi:10.3732/apps.1300070.
[23] George, A. (2018). Simple and efficient method for functional RNA extraction from tropical medicinal plants rich in secondary metabolites. *Tropical Plant Research*, *5*, 8-13. Doi:10.22271/tpr.2018.v5.i1.002.
[24] Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K. (2001). Quantitation of DNA and RNA with absorption and fluorescence spectroscopy. Doi:10.1002/0471142727.mba03ds76.
[25] Koetsier, G., Cantor, E. (2019). A practical guide to analyzing nucleic acid concentration and purity with microvolume spectrophotometers. New England Biolabs Inc, 1-8.
[26] Sah, S. K., Kaur, G., Kaur, A. (2014). Rapid and reliable method of high-quality RNA extraction from diverse plants. *American Journal of Plant Sciences*, *05*, 3129-3139. Doi:10.4236/ajps.2014.521329.
[27] Inglis, P. W., Pappas, M. D. C., R. Resende, L. V., Grattapaglia, D. (2018). Fast and inexpensive protocols for consistent extraction of high quality DNA and RNA from challenging plant and fungal samples for high-throughput SNP genotyping and sequencing applications. *PLOS ONE, 13*, e0206085. Doi:10.1371/journal.pone.0206085.
[28] Kanani, P., Shukla, Y. M., Modi, A. R., Subhash, N., Kumar, S. 2019. Standardization of an efficient protocol for isolation of RNA from *Cuminum cyminum*. *J King Saud Univ Sci. 31*, 1202-1207. Doi: 10.1016/j.jksus.2018.12.008.
[29] Uchida, S., Adams, J. C. (2019). Physiological roles of non-coding RNAs. *Am J Physiol Cell Physiol, 317*, C1-C2. Doi:10.1152/ajpcell.00114.2019.
[30] Skrypina, N. A., Timofeeva, A. V., Khaspekov, G. L., Savochkina, L. P. (2003). Beabealashvilli, R. Total RNA suitable for molecular biology analysis. *J Biotechnol*, *105*, 1-9. Doi:10.1016/s0168-1656(03)00140-8.
[31] Ueno, D., Yamasaki, S., Kato, K. (2022). Methods for detecting RNA degradation intermediates in plants. *Plant Sci. 318*, 111241. Doi:10.1016/j.plantsci.2022.111241.
[32] Zhang, N., Hu, G., Myers, T. G., Williamson, P. R. (2019). Protocols for the analysis of microRNA expression, biogenesis, and function in immune cells. *Curr Protoc Immunol*, *126*, e78. Doi:10.1002/cpim.78.
[33] Van Wynsberghe, P. M., Chan, S. P., Slack, F. J., Pasquinelli, A. E. (2011). Analysis of microRNA expression and function. *Methods Cell Biol*, *106*, 219-252. Doi:10.1016/B978-0-12-544172-8.00008-6.
[34] Annese, T., Tamma, R., De Giorgis, M., Ribatti, D. (2020). microRNAs biogenesis, functions and role in tumor angiogenesis. *Front Oncol 10*, 581007. Doi:10.3389/fonc.2020.581007.
[35] Pagano, L., Rossi, R., Paesano, L., Marmiroli, N., Marmiroli, M. (2021). miRNA regulation and stress adaptation in plants. *Environmental and Experimental Botany*, *184*. Doi:10.1016/j.envexpbot.2020.104369.
[36] Gillespie, P., Ladame, S., O'Hare, D. (2019). Molecular methods in electrochemical microRNA detection. *The Analyst, 144*, 114-129. Doi:10.1039/c8an01572d.
[37] Ouyang, T., Liu, Z., Han, Z., Ge, Q. (2019). MicroRNA detection specificity: Recent advances and future perspective. *Anal Chem*, *91*, 3179-3186. Doi:10.1021/acs.analchem.8b05909.
[38] Cheng, Y., Dong, L., Zhang, J., Zhao, Y., Li, Z. (2018). Recent advances in microRNA detection. *Analyst*, *143*, 1758-1774. Doi:10.1039/C7AN02001E.
[39] Saddhe, A. A., Malvankar, M. R., Kumar, K. (2018). Selection of reference genes for quantitative real-time PCR analysis in halophytic plant *Rhizophora apiculata*. *PeerJ*, *6*, e5226. Doi:10.7717/peerj.5226.
[40] Shukla, P., Reddy, R. A., Ponnuvel, K. M., Rohela, G. K., Shabnam, A. A., Ghosh, M. K., Mishra, R. K. (2019). Selection of suitable reference genes for quantitative real-time PCR gene expression analysis in Mulberry (*Morus alba* L.) under different abiotic stresses. *Molecular Biology Reports*, *46*, 1809-1817, doi:10.1007/s11033-019-04631-y.
[41] Joseph, J. T., Poolakkalody, N. J., Shah, J. M. (2018). Plant reference genes for development and stress response studies. *Journal of Biosciences*, *43*, 173-187. Doi:10.1007/s12038-017-9728-z.
[42] Ferreira, M. J., Silva, J., Pinto, S. C., Coimbra, S. I. (2023). Choose you: Selecting accurate reference genes for qPCR expression analysis in reproductive tissues in *Arabidopsis thaliana*. *Biomolecules*, *13*, 463. Doi:10.3390/biom13030463.
[43] Vaccaro, M., Bernal, V. O., Malafronte, N., De Tommasi, N., Leone, A. (2019). High yield of bioactive abietane diterpenes in *Salvia sclarea* hairy roots by overexpressing cyanobacterial DXS or DXR genes. *Planta Medica, 85*, 973-980.
[44] Liu, Y., Yang, Z.-m., Xue, Z.-l., Qian, S.-h., Wang, Z., Hu, L.-x., Wang, J., Zhu, H., Ding, X.-m., Yu, F. (2018). Influence of site-directed mutagenesis of UbiA, overexpression of dxr, menA and ubiE, and supplementation with precursors on menaquinone production in Elizabethkingia meningoseptica. *Process Biochemistry*, *68*, 64-72. Doi:10.1016/j.procbio.2018.01.022.
[45] Salamon, S., Zok, J., Gromadzka, K., Blaszczyk, L. (2021). Expression patterns of miR398, miR167, and miR159 in the Interaction between bread wheat (*Triticum aestivum* L.) and pathogenic *Fusarium culmorum* and beneficial *Trichoderma* Fungi. *Pathogens*, *10*. Doi:10.3390/pathogens10111461.
[46] Leng, X., Wang, P., Zhu, X., Li, X., Zheng, T., Shangguan, L., Fang, J. (2017). Ectopic expression of CSD1 and CSD2 targeting genes of miR398 in grapevine is associated with oxidative stress tolerance. *Funct Integr Genomics*, *17*, 697-710. Doi:10.1007/s10142-017-0565-9.
[47] Sun, Z., Shu, L., Zhang, W., Wang, Z. (2020). Cca-miR398 increases copper sulfate stress sensitivity via the regulation of *CSD* mRNA transcription levels in transgenic. *Arabidopsis thaliana PeerJ., 8*, e9105, Doi:10.7717/peerj.9105.

[48] Li, J., Song, Q., Zuo, Z.-F., Liu, L. (2022). MicroRNA398: A master regulator of plant development and stress responses. *International Journal of Molecular Sciences*, *23*, 10803. Doi:10.3390/ijms231810803.