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Identification of miR414 and Its Regulatory Roles in Terpenoid Biosynthesis in *Mentha* spp.

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Abstract
Mentha spp. (mints), belonging to the *Lamiaceae* family, are aromatic, perennial, herbaceous, and rhizomatous plants. They produce terpenoids as secondary metabolites. Terpenoids application include as fragrance, flavours, pharmaceutical agents, and insecticides. From previous study, miR414 has been found involved in the regulation of TPS21. Previous research has indicated the involvement of miR414 in secondary metabolic pathways in mint plants. However, it just a computational approach, experimental is needed to validate the relationship between miR414 and TPS21. This study focused on the identification of miR414 and its regulatory roles in terpenoid biosynthesis in *Mentha* spp.. The objectives of this study are: (i) to extract RNA from control and treated plants by using PureLink™ PlantRNA Reagent, (ii) to identify the stem-loop structure of mint plants through reverse transcriptase PCR and (iii) to analyze the expression of miRNA toward the target gene in control and treated plants through reverse transcriptase PCR. This experiment was carried out to verify the behaviour of miR414 towards its target, TPS21 under stress conditions which known as wounding. The experimental steps included RNA extraction, with subsequent checks performed by Nanodrop, and RNA integrity by gel electrophoresis. Primer sets of interest were designed through software Primer 3 and RT-PCR was done to amplify DNA fragments of interest. By using gel electrophoresis, the results showed the stemloop structure identification of miR414 was in a band of 151 base pairs, indicating the presence of the miR414 stem-loop structure. The gene expression was analysed using Image J. The expression value was then calculated. The expression value for miR414 were 1.203 in control sample and 1.280 in treated sample. While the expression value of TPS 21 were 0.697 in control sample and 1.381 in treated sample. Analysis of gene expression revealed slight changes in miR414 expression, while TPS21 expression almost doubled under wounding conditions. These results indicates that miR414 might not directly play roles in the response to wounding stress conditions. While terpenoid has play important in the mint plants' defence mechanism, thus more TPS21 is needed to produce more terpenoid. Overall, this study provides insights into the regulatory roles of miR414 in terpenoid biosynthesis, shedding light on the molecular mechanisms involved in mint plants.
Keywords: *Mentha* spp,; miR414; TPS 21; wounding stress; regulatory roles

Introduction

Mentha is a famous genus (in the *Lamiaceae* family) that possesses both medicinal and fragrance value. There are 25–30 species in *Mentha* genus including three main origin species including *Mentha spicata, Mentha canadensis, and Mentha aquatica* (Alu'datt et al, 2018). The biosynthetic pathways for the essential oils produced by *Mentha* plants, the majority of which are terpene and terpenoids, are largely understood. Terpene that are derived from *Mentha* spp. have been utilized by humans for a variety of things, such as pesticides, flavourings, and scents. Terpene compounds are extremely valuable commercially, but they also serve vital biological purposes in plants. Gibberellin phytohormones, for example, are terpene metabolites that play an important role in the variety of mechanisms that plants interact with their environment and are crucial for plant growth and development (Malik et al, 2022).

In nature, terpene synthases are enzymes that produce terpenes (TPS). TPS uses complex chemistry, including cyclizations, methyl-migration, rearrangements, as well as proton and hydrogen transfers, to produce different types of terpenes that serve as the building blocks for more than 80,000 distinct terpenoids. (Raz et al, 2020). Terpenoids, phenolic compounds, alkaloids, and chemicals containing sulphur are the four main groups of secondary plant metabolites. Terpenoid is the secondary metabolite on which this study focuses. Terpene synthase (TPS) is an enzyme that can catalyze the production of terpenoids. Terpenoids display a wide spectrum of biological activity against a number of diseases or infections, including viral and bacterial infections, cancer, malaria, inflammation, and tuberculosis.

MicroRNAs (miRNAs) are a class of non-coding RNAs that play significant roles in regulating gene expression. Most miRNAs are produced as a result of DNA sequences being translated into primary, precursor, and mature miRNAs. (O'Brien et al., 2018). The miRNA serves as a guide and inhibits the production of the target mRNA by base-pairing (MacFarlane & Murphy, 2010). The stem loops of plant miRNA precursors often have longer stem than those of animal pre-miRNAs, and their structural diversity is fairly wide. (Wahid et al, 2010) Pre-miRNAs have secondary structures that resemble hairpins. Asymmetric bulges are frequently formed by unpaired groupings of nucleotide bases. For plant miRNAs, the minimum size of these bulges is crucial since it indicates the integrity of the structure.

MiRNAs regulate the expression of genes encoding transcription factors, proteins that respond to stress, and other proteins that affect biological processes in plants. Some of the biological processes that miRNAs regulate include the maintenance of genome integrity, primary and secondary metabolism, development, signal transduction, signalling pathways, homeostasis, innate immunity, and adaptive responses to biotic and abiotic stress (Djami-Tchatchou et al, 2017). It is well known that miRNAs regulate a number of post-transcriptional biological processes. Presently, some studies demonstrate the function of miRNAs in the control of secondary metabolic pathways (Gupta et al, 2017). As a result, miRNAs can be used to control the generation of chemicals obtained from secondary metabolism (Marcela et al, 2019).

Materials and methods

Plantation and treatment

About 40-day-old plants with roughly identical size were selected and purchased for the experiment. Wounding treatment is chosen in this experiment. In order to mechanically wound leaves, at least five completely extended leaves were punctured (Figure 3.1). Plants were left in the growth room for a further 48 hours after damage treatments to allow damage-induced reactions to take place (Tang et al, 2012). A control plant was prepared (non-wounding condition).

Figure 3.1 Wounding treatment

Sample Preparation

Both control and treated leaves were grinded under presence of liquid nitrogen. By using a cold mortar and pestle, grinds the leaves until it become fine powder. The samples were stored at -80°C, prior to avoid RNA degradation. Meanwhile, cool the RNase-free microcentrifuge tubes and kept cold in ice, for the extraction purpose (Gismondi et al, 2017).

RNA Extraction

About 0.1 gram of frozen, powdered plant tissue was mixed with 0.5 mL of cold (4°C) PureLink® Plant RNA Reagent. The material is thoroughly resuspended after being briefly vortexed or flicked at the tube's bottom to mix it. The tube was incubated at room temperature for 5 minutes. To maximize surface area during incubation, lay the tube down horizontally. The solution was then clarified

bycentrifuging it at 12,000 g for 2 minutes at room temperature in a microcentrifuge. A fresh, RNasefree tube was used to transfer the supernatant. The supernatant was transferred to a neat RNase-free tube. 0.1 mL of 5 M NaCl was added to the clarified extract. By tapping the tube, combine. To the sample, add 0.3 mL of chloroform. Invert the tube to completely mix. The phases were separated by centrifuging the material at 12,000 g for 10 minutes at 4°C. The aqueous upper phase was transferred to a tidy, RNase-free tube. Isopropyl alcohol should be added in an equal volume to the aqueous phase. After that, combine and let it rest for 10 minutes at room temperature. The sample was centrifuged at 12,000 g for 10 minutes at 4 °C. To the particle, add 1 mL of 75% ethanol after decanting the supernatant while being careful not to lose the pellet. At room temperature, centrifuge at 12,000 g for 1 minute. Decant the supernatant with care, being careful not to lose the particle. Briefly centrifuged to gather the leftover liquid, then pipetted it out. To the RNA pellet, 10- 30 mL of RNase-free water was added. Re-suspended the RNA by pipetting the liquid up and down over the pellet. If any cloudiness is observed, centrifuged the mixture at room temperature for 1 minute at 12,000 g, and then pour the RNA-containing supernatant into a fresh tube. At -80°C, keep pure RNA (ThermoFisher Scientific, USA).

Evaluation of RNA Concentration, Purity and Integrity

The main objective of quality control measures is to ensure the RNA integrity. After that, proceed with the next step if the RNA integrity is good enough. Nanodrop spectrophotometer (ND-1000) was used for the quantification of the total RNA samples. Absorbance at both 260nm and 280nm were observed to assess the purity of the RNA samples. The 260/230 ratio can also be employed to measure the purity of RNA samples and the present of contaminants (Garcia-Alegria et al, 2020).

RNA integrity was evaluated using electrophoresis on a 1% agarose gel.Usually, the 28S and 18S ribosomal RNA bands may be observed on the gel due to their high molecular weights and great abundance in tissues. RNA that is intact is indicated by sharp, distinct bands (Hammad, 2023). The gels were placed in mini-gel electrophoresis apparatuses before it is soaked completely with 1x TAE buffer. The samples consist of 1x DNA Loading buffer, prepared from a 10x DNA Loading buffer stock (1.9 mM xylene cyanol, 1.5 mM bromophenol blue, 25% glycerol in sterile dH2O) and 1 µg of total RNA isolated from *Mentha* was loaded into the each well. The gels were be run for approximately 60 minutes with constant voltage (45V). Then, the gel image was captured under UVI transillumination (Aranda et al). Intact total RNA will show sharp and clear bands on the gel, whereas partially degraded RNA form smeared bands, and completely degraded shows very low molecular weight smeared appearance of the bands. A 2:1 ratio (285:18S) is a good sign of a completely intact RNA sample.

Primer Design

Primer design is done by using Primer 3. Searched the required nucleotide sequence, selected the target sequence from NCBI nucleotide and saved it in FASTA format in a text file. Opened the Primer3 (version 4) software. Pasted the nucleotide sequence (in FASTA format) in the box for the source sequence on the Primer 3 page. Set the required parameter. Best choice of primer sets was selected. After the primer design, PCR is then carried out by using NEBNext PCR kits. Table 3.1 showed the details of the designed primer sets.

Table 3.1 Details on designed primer sets

Primer Types	Primer Sequences	Primer Melting Temperature/ Tm (° C)	GC Content (%)
Tubulin *Mentha*_F	5'-AAG TCG GAAATG CTT GCT GG-3'	56.1	50.0
Tubulin *Mentha*_R	5'-AGC ATC ATC ACC TCC ACC AA-3'	56.4	50.0
miR414_F	5'-TCA GAT GCC TCA AAT GGA TCA- 3'	53.9	42.9
miR414_R	5'-GGC AAC TGG TCC ATG ATA TCT G-3'	55.5	50.0
TPS21_F	5'-TCC GCC AAA TAC CAT CCA AC-3'	55.4	50.0
TPS21_R	5'-GGC GCT GGA TTG TAT CGA TG-3'	56.2	55.0

cDNA synthesis

cDNA synthesis was started by preparation of the sample. RNA serves as the template in cDNA synthesis. Trace amounts of genomic DNA (gDNA) may be co purified with RNA. Double-strand specific DNases are added to remove the gDNA. After preparing the RNA samples and removing the DNA from the samples and also the container, the reaction mix for cDNA were prepared. The reaction mix involves RNA template, buffer, dNTPs, DTT, RNase inhibitor, and RNase-free water. The reverse transcription reaction is then carried out through three fundamental processes: primer annealing, DNA polymerization, and enzyme deactivation. The kit used in this experiment was RevertAid First Strand cDNA Synthesis Kit. The protocols were concluded in the Table 3.2.

Table 3.2 Protocol for a cDNA synthesis

	Control Sample (µL)	Treated Sample (µL)
Template RNA	1.0	2.0
Primer	1.0	1.0
Nuclear-free Water	10.0	9.0
Total	**12.0 µL**	
5X Reaction Buffer	4.0	
RiboLock RNase Inhibitor (20 U/ µL)	1.0	
10 mM dNTP	2.0	
RevertAid M-MuLV RT (200 U/ µL)	1.0	
Total	**20 µL**	

There were differences in thermostability among reverse transcriptase. Using a thermostable reverse transcriptase allows a higher reaction temperature. Suitable reverse transcriptase was then chosen and added to isolate the cDNA. In example, a thermostable reverse transcriptase able to denature RNA with GC content or secondary structures without impacting the enzyme activity.

RT-PCR for miR414 and TPS 21

Reverse transcriptase PCR (RT PCR) was conducted using NEBNext® High Fidelity 2X PCR Master Mix (New England Biolabs) to validate the existence and expression of those miRNAs and target genes (TPS21) involved in the terpenoid pathway under the treatment. The reaction was occurred in three phases which are denaturation, annealing, and extension. An initial denaturation was set at 94 ° C for 30 seconds. This duration is sufficient for most amplicons from pure DNA templates. For templates that need it, longer denaturation times can be employed. During thermocycling, the denaturation stage should be minimized. A 30 seconds denaturation at 94 ° C is recommended for most templates. In the

annealing phase, use a 30 second annealing step at 3 ° C above the primer melting temperature (Tm) of the lower Tm primer (Table 3.4). The ideal annealing temperature for each primer combination can also be achieved via a temperature gradient. To note, the annealing temperature for the reaction was optimized based on the primers designed. The recommended temperature for the extension phase is 68 ° C. For cDNA or large, complicated templates, the extension time can be raised to 40 seconds per kb if necessary. The protocol advised a last extension of 5 minutes at 68 ° C. The PCR product was run on 3% agarose gel electrophoresis, using SYBR Green nucleic acid gel strain. These protocols and thermocycling condition for a routine PCR were concluded in Table 3.3 and 3.4. While Table 3.5 showed the annealing temperature of different genes. Figure 3.2 showed the gel electrophoresis.

Table 3.3: Protocol for a routine PCR

Components	Volume (µL)
One Taq Quick-Load 2X Master Mix with Standard Buffer	12.5
10 µM Forward Primer	0.5
10 µM Reverse Primer	0.5
Template DNA	1.0
Nuclear-free Water	10.5
Total	**25 µL**

Table 3.4: Thermocycling condition for a routine PCR

Steps	Temperature (° C)	Time
Initial Denaturation	94.0	30 sec
35 cycles	94.0	30 sec
	Annealing	1 mins
	68.0	
Final Extension	68.0	5 mins
Hold	10.0	∞

Table 3.5: Annealing temperature of different genes

Gene of Interest	Annealing Temperature (° C)
Tubulin *Mentha*	51
miR414	50
TPS21	50
Negative Control	51

Figure 3.2 Gel electrophoresis

Gene expression analysis for miR414 and TPS 21

The analysis of TPS 21 and miR414 expression of control and treated plants were compared by using image J. The gel image of the target gene and miR414 of both control plants were compared visually by using the software. Comparison was made on the bands appeared on the gel. Peak areas were first calculated and then normalized with reference genes to calculate a validate expression value.

Results and discussion

Evaluating RNA Concentration, Purity, and Integrity using thePlant

RNA Reagent from the Mint SamplesIt is essential to measure the quantity and quality of purified RNA in order to decide the volume of each sample that needed to utilize in subsequent application such as reverse transcription or RT-PCR. It utilizes the principle of UV-Vis spectroscopy to measure the absorbance of a sample at specific wavelengths of light. The Nanodrop spectrophotometer is usually used to measure the concentration and purity of nucleic acid samples, including RNA (Hayford et al, 2022). There are two key parameters considered: (i) the concentration and (ii) the purity ratios when evaluating the data from a Nanodrop measurement. The concentration of RNA in the sample was reported in the units of ng/µL (nanograms per microliter) in this research. According to the Nanodrop spectrophotometer, the concentration of RNA indicates the amount of RNA present in the sample (Hammad, 2023).

The purity ratios showed information about the quality of the RNA sample by assessing the presence of contaminants such as protein, phenol, organic compounds or other impurities that can bring effects in downstream applications (Farrel, 2023). The two commonly used ratios are: (i) 260/280 and (ii) 260/230. The ratio of absorbance at 260 nm and 280 nm (260/280 ratio) is used as an indicator of RNA purity as nucleic acids and proteins have absorbance maxima at 260 and 280 nm, respectively (Kenney et al, 2019). 260/280 ratios of ~2.0 are often considered to be "pure" for RNA. While evaluating probable contaminants, the 260/230 ratio, which measures absorbance at 260 nm and 230 nm, is utilized. The "pure" nucleic acid 260/230 readings are usually higher than the equivalent 260/280 values. The expected readings of 260/230 typically range from 2.0 to 2.2. (ThermoFisher Scientific, USA). A high 260/230 ratio shows a clean RNA sample with less or no contamination (Hammad, 2023). It is used to determine the presence of contaminants such as salts, phenol, or other organic compounds.

Table 4.1: Results of Nanodrop Spectrophotometer

Parameter	Control Sample	Treated Sample
Concentration (ng/µL)	1994.7	1166.2
260/280	2.07	2.16
260/230	1.67	1.79

Based on Table 4.1, the results for the ratio 260/280 for both control and treated samples are out of the restricted range which are 2.07 and 2.16 respectively. When the 260/280 ratio is anomalous, which occurs when the ratio is generally low, this indicated that the sample is probably contaminated by phenol, guanidine, or another reagent used in the extraction procedure. Inaccurate ratios have also been seen at a very low nucleic acid concentrations (10 ng/l) (Thermo Fisher Scientific, USA). While for the 260/230 ratio, both control and treated samples were below 2.0 which are 1.67 and 1.79 respectively. This indicates that there may be contamination from substances that absorb light at 230

nm, such as residual phenol or other organic compounds commonly found in plant samples. These contaminants can interfere with downstream applications, such as reverse transcription, cDNA synthesis, or enzymatic reactions. From the results, it can be assumed that the extracted RNA is considered as a moderate quality RNA.

There are several reasons that can cause the contamination of the extracted RNA (eg. sample contamination, RNase contamination etc). Sample contamination can be one of the reasons that affect the quality of the extracted RNA. The contaminants present in the biological sample used for RNA extraction can interfere with the purity of the extracted RNA (Toni et al, 2018). This can include DNA, proteins, salts, organic compounds, and other cellular debris. In addition, RNases are enzymes that can degrade RNA molecules. If the RNA extraction process is not carried out under RNase-free conditions, these enzymes can contaminate the sample and degrade the RNA, leading to reduced yield and quality. RNases can be introduced through skin contact, aerosols, dust particles, or contaminated equipment (New England Biolabs).

To increase the quality and purity of extracted RNA, we can take the initiative to perform additional purification steps. If the 260/230 ratio remains low even after optimizing the extraction protocol, we can consider performing additional purification steps, such as isopropanol precipitation or column-based purification methods, to remove contaminants (Toni et al, 2018). Besides, we can also try to dilute the sample. In some cases, diluting the RNA sample with RNase-free water or a suitable buffer can help improve the 260/230 ratio. However, be cautious with dilution, as it may also lower the RNA concentration.

The Nanodrop software provides several parameters to assess the quality of RNA. It's important to note that while the Nanodrop provides a quick and convenient method for RNA quantification and purity assessment, it cannot provide detailed information about the RNA integrity or the presence of specific contaminants. For more comprehensive analyses, techniques such as gel electrophoresis or RNA sequencing may be required (Farrell, 2023).

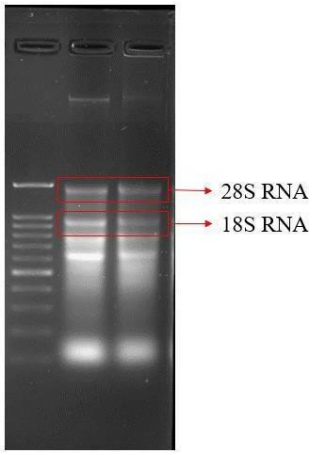

Figure 4.1 Gel electrophoresis for evaluation of the integrity of RNA extracted from the mint plant. (L1: ladder, L2: control sample, L3: treated sample)

RNA integrity was then evaluated using electrophoresis on a 1% agarose gel (Figure 4.1). The presence of gDNA was observed. This is because gDNA does not removed before convert the RNA sample intocDNA. To avoid this, gDNA removal kit such as RapidOut DNA Removal Kit can be used to remove thegDNA. (Thermo Fisher, USA) In most cases, RNA integrity may be evaluated quickly and easily using agarose gel electrophoresis (Hasan et al, 2021). From the Figure 4.1, there are a total of six bands observed in the gel. The first band is out of range of the ladder indicates that there is the presence of genomic DNA (gDNA). This is because there is no additional step to remove the gDNA from the samples before the integrity check. The presence of gDNA in the samples can be negligible because gDNA will not be involved in the reverse transcription of the RNA sample into cDNA. Nevertheless, it is good to remove the gDNA to improve the purity of the RNA sample.

On gel pictures displaying the 28S and 18S rRNA together with the other bands that may represent short RNAs. Due to their enormous molecular weights and widespread distribution in tissues, 28S and 18S rRNA are present in tissues.(Hammad, 2023). The RNA solutions are intact if there is no smearing underneath the two distinct, crisp bands. (Hasan et al, 2020). Theoretically, the quality of RNA is considered high if the ratio of 28S:18S bands is 2.0. Yet, the ratio cannot be observed from the results. This is because of the presence of contaminants as the results shown in Nanodrop. Gel Electrophoresis for the Identification of Stem-loop Structure in the Mint Samples

According to Singh et al., (2016), pre-miRNAs have secondary structures that resemble hairpins. Asymmetric bulges are frequently formed by unpaired groupings of nucleotide bases. The stability of the structure is represented by the minimum size of these bulges, which is a crucial characteristic for plant miRNAs. Particularly inside the miRNA/miRNA* duplex, bulges should be small (one or two bases) and infrequent (usually one or less). It is known that miRNAs have evolved to be conserved across plant species. Given that miR414 is conserved across a variety of dicots and monocots, including Arabidopsis, it was chosen in this study for experimental validation (Guleria & Yadav, 2011).

The amplified DNA fragments produced during the PCR process can be visualized by gel electrophoresis. Gel electrophoresis separates the DNA fragments based on their size. 3% of agarose gel was used in this experiment, creating a molecular sieve through which the DNA molecules migrate when an electric field is applied. Smaller DNA fragments move more easily through the gel matrix, resulting in separation according to size. By comparing the migration of the DNA fragments with known molecular weight markers (DNA ladder), the approximate size of the PCR products can be determined. The stem-loop structure is of significant importance in miRNA molecules. miRNAs are initially transcribed as long primary transcripts called pri-miRNAs. These pri-miRNAs undergo a series of processing steps to generate mature miRNAs (Rehfeld et al, 2014). The stem-loop structure is critical in the formation of pre-miRNAs. Within the pri-miRNA molecule, the stem-loop structure folds back on itself, forming a hairpin structure (Georgakilas et al, 2018). This hairpin structure serves as a recognition site for enzymes involved in processing and maturation, such as Drosha and Dicer.

After the mature miRNA has been loaded onto the RNA-induced silencing complex (RISC), it directs the RISC to the messenger RNA (mRNA) molecules it needs to target by base-pairing with the mRNA's complementary sequence (Lelandais-Briere et al, 2010). The stem-loop structure of miRNA is vital in order to determine the target specificity. The sequence and length of the stem and loop regions influence the binding affinity and specificity of the miRNA to its target mRNA. Changes in the stem-loop structure can potentially alter target recognition and affect the regulatory functions of miRNAs.

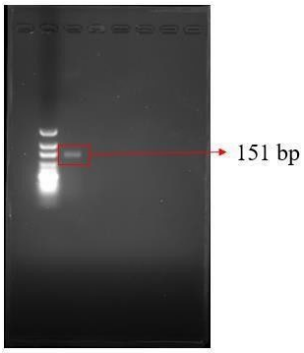

Figure 4.2 Gel Electrophoresis for Identification of miR414 Stem-loop Structure in Mint Plants. (L2: ultra-low ladder, L3: control sample)

Figure 4.2 shows the results of gel electrophoresis for identification of miR414 stem-loop structure in mint plants. From Figure 4.2, a band with 151 bp was observed. This indicates that there is presence of step-loop structure in miR414. In addition, the observed band can also be used to validate that miR414 was successfully extracted from the mints sample (Objective 1). Another way to validate the successful extraction of RNA are sequencing or specific probes. These techniques can be used to determine the exact nucleotide sequence of the extracted RNA fragment. In the present study, miR414 was observed to have one bulge. The observed band validates the result of the previous in silico prediction made by Singh et al., (2016). The annealing temperature used for the identification of stem-loop structure in mint plants is 50 °C.

The number and intensity of bands observed in PCR can vary with the annealing temperature. A numerous bands are seen with greater discernibility as the annealing temperature raised (Korvin et al, 2014). The annealing temperature affects the specificity of PCR. The annealing temperature is resolute by calculating the melting temperature (T_m) of the selected primers for PCR amplification. These ensures that the primers anneal specifically to the target DNA and not to non-

specific regions (ThermoFisher Scientific, USA). The optimal annealing temperature can be determined by using a gradient PCR method.

Analysis of Gene Expression for both miR414 and TPS21 in Control and Treated Sample

Reference genes are used as internal controls or normalization factors to ensure accurate gene expression analysis in various applications, such as gene expression profiling, qPCR, and Western blotting. The right reference genes must be found for PCR in order to normalize gene expression and prevent errors brought on by variations in experimental protocols. Thus, normalization of expression values requires a good reference gene with consistent expression under experimental conditions (Soni et al, 2021). Typically, a reference gene should be expressed in cells and tissues over the course of all physiological situations (Yang et al, 2023). Erroneous experimental outcomes may result from variations in reference genes. Since no gene has been found to express uniformly in all cells under all physiological and experimental circumstances, empirical validation of stable reference genes is still necessary to precisely gauge the expression of genes of interest (Fischer et al, 2022). The reference chosen in this experiment is Tubulin. Tubulin has been used as a reference gene for miRNAs and TPS 21 in previousstudies by Liang et al., (2018) and Yahyaa et al., (2015) respectively.

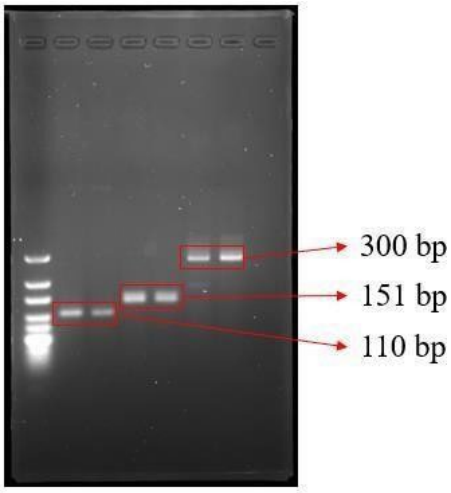

Figure 4.3 Gel Electrophoresis for Gene Expression of miR414 and TPS21 in Both Control and Treated Samples

(L1: ultra low ladder, L2: Tubulin *Mentha*-Control, L3: Tubulin *Mentha*-Treated, L4: miR414-Control, L5: miR414-Treated, L6: TPS21-Control, L7: TPS21-Treated, L8: Negative Control)

Based on Figure 4.3, the observed bands indicate that the genes of interest were expressed in mint plants. The sizes of bands for tubulin *Mentha*, miR414 and TPS21 are 300 bp, 151 bp and 110 bp respectively. These results were then analyzed by using bioinformatic tools, Image J.

Table 4.2: Results on peak area for gene expression analyzing through Image J

Genes of Interest	Peak Area	
	Control	Treated
Tubulin *Mentha*	10576.045	7309.530
miR414	12723.250	9358.300
TPS21	7374.894	10093.087

Table 4.3: Results on expression value

Genes of Interest	Expression Values	
	Control	Treated

Tubulin *Mentha*	1.00	1.00
miR414	1.203	1.280
TPS21	0.697	1.381

The peak area was counted in Image J by measuring the intensity of the bands. The data was then normalized into expression values. From the tables, results showed that the expression value of miR414 was slightly changed in the treated sample (wounding condition). These results indicate that miR414 might not be directly involved in the response in the wounding stress. According to Sun et al., (2012), different miRNAs may play distinct roles in the response to specific stress conditions. This led to slight changes in the gene expression of miR414 under wounding stress. While the expression value of TPS21 increased dramatically, which increased almost double in the treated sample (wounding condition). The dramatic changes indicate that terpenoids play an important role in the mint plant defense mechanism.

Terpenoids, including monoterpenes, are a group of secondary metabolitesproduced by plants that play a crucial role in plant defense mechanisms against biotic and abiotic stresses (González et al, 2023). Terpenoids are involved in both direct and indirect defense mechanisms. Terpenoids can act as directdefense mechanisms against insect pests by exerting anti-insectan activity. In previous studies, some methylcyclopentanoid monoterpenes contained in Teucrium marum have been shown to have anti- insectan activity (Firoznezhad et al, 2022). Terpenoids can also act as indirect defense mechanisms by attracting insect carnivores that feed on herbivores. These volatiles induce defense responses in the systemic parts of the same plant and attract insect carnivores that feed on the herbivores, thereby reducing the damage caused by herbivorous insects (Sharma et al, 2017). Theoretically, the gene expression of miR414 and TPS21 should be inversely proportional as the results that predicted through computational approach in the previous studies. However, the results in this experiment obeyed the theory. This might include several reasons: (i) the reference gene used is not suitable enough, (ii) TPS21 is not mainly regulated by miR414 and, (iii) degradation of some portion of miR414 happened. To validate the reasons, further studies are needed.

Conclusion

In conclusion, the experimental procedures conducted in this study involved multiple steps and analyses to investigate RNA extraction, stem-loop structure identification, gel electrophoresis, and gene expression analysis. Firstly, RNA extraction was successfully performed, and the quality of the extracted RNA was assessed through concentration measurements and the ratios of 260/230 and 260/280. Additionally, the integrity of the RNA samples was verified. Moving on to stem-loop structure identification, this process was carried out successfully. Moreover, gel electrophoresis was performed, revealing a band at 151 base pairs. The subsequent focus of the research was the analysis of gene expression. It was observed that under stress conditions, the gene expression of both miR414 slightly changed, while the gene expression of TPS 21 nearly doubled. This finding suggests that stress has an impact on the expression levels of these genes. Considering future applications, the study proposes genetic manipulation techniques to modulate terpenoid production in mints. By manipulating the genes involved, it may be possible to increase or decrease terpenoid production, potentially benefiting the field of natural product synthesis.

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