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Overexpression of amiR2937 and amiR854e in Transgenic *Arabidopsis thaliana* Indirectly Impacts the Photosynthesis Performances by Targeting Specific Target Transcripts in the MEP Pathway

Tuan Aini Nadirah Che-Wan-Ngah[1], Muhamad Hafiz Che Othman[2] and Ismanizan Ismail[1,2]*

[1]Institute of System Biology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
[2]Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

ABSTRACT

Artificial miRNAs (amiRNAs) are artificial small RNAs engineered to silence specific plant mRNA transcripts. They are generated by expressing a functional microRNA (miRNA) with modified sequences *in planta*. Two miRNAs, miR2937 and miR854e, were selected based on their predicted target transcript, *GGPS2* (geranylgeranyl pyrophosphate synthase 2) and *TPS13* (terpenoid synthase 13). In the methylerythritol phosphate pathways, *GGPS2* and *TPS13* enzymes play a role in synthesizing sesquiterpenes, triterpenes, diterpenoids, carotenoids, gibberellins, and chlorophyll, respectively. Therefore, in this study, these two miRNAs were overexpressed in *Arabidopsis thaliana* in single and co-overexpression to analyze the change in the abundance of phytol and trans-beta-lone compounds. Through real-time quantitative polymerase chain reaction (RT-qPCR) analysis, a fold-up regulation of amiR2937 and amiR854e was observed in both transgenic plants harboring single and double constructs. Meanwhile, the *GGPS2* and *TPS13* enzymes showed a decreasing pattern in all transgenic plants, indicating that the miRNAs had successfully suppressed the target transcripts. Solid-phase microextraction-gas chromatography-mass spectrometry analysis revealed that the number of phytols was decreased in all transgenic plants but was significant in plants harboring construct miR854e. Meanwhile, there is an increasing pattern of trans-beta-ionone in all transgenic plants

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E-mail addresses:
aininadirah56@gmail.com (Tuan Aini Nadirah Che-Wan-Ngah)
hafiz87@ukm.edu.my (Muhamad Hafiz Che Othman)
maniz@ukm.edu.my (Ismanizan Ismail)
*Corresponding author

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compared to wild-type plants. Consistently, with the decrease in phytol content, soil plant analysis development value, and total chlorophyll content, the photosynthesis rate decreased in the transgenic plants compared to the wild type. Indeed, the overexpression of these two miRNAs affects the production of target transcript and changes the plant development.

Keywords: amiRNAs, *Arabidopsis thaliana*, gene-silencing, post-transcriptional regulation, sesquiterpenoid and phytol biosynthesis

INTRODUCTION

In plants, metabolites from both primary and secondary metabolism play significant roles in development and growth. The primary metabolism of plants produces important molecules: carbohydrates, lipids, proteins, and nucleic acids, essential metabolites for general plant development and growth. In contrast, the secondary metabolism of plants produces many small biomolecules known as secondary metabolites (SMs). These SMs include phenolics, phytols, terpenoids, nitrogen-containing compounds, oligosaccharides, and alkaloids. In addition, SMs have been classified into terpenoids, phenylpropanoids, and alkaloids based on their biosynthetic pathway site and natural chemical properties. However, the production of SMs in the plant is very low, and the plant releases the compound only when needed. The plant species affect how secondary metabolites are produced and also on certain conditions such as weather, environmental stress, and nutritional sources (Akula & Ravishankar, 2011; Ramirez-Estrada et al., 2016).

Artificial microRNA (amiRNAs) are artificial small RNAs (sRNAs) engineered to silence specific plant mRNA transcripts. The expression of a functional miRNA with modified sequences *in planta* generates them. They are derived from the Dicer-Like1 (DCL1) protein cleavage of miRNA precursors with fold-back structures. MicroRNA (miRNAs) are short (19–24 nucleotides) non-coding RNAs influencing key plant processes like growth, development, and stress response (Ameres & Zamore, 2013; Martin et al., 2010). Research on miRNA biogenesis laid the foundation for amiRNA design, and in recent years, there has been a proliferation of new information on the role of sequence and structure in pri-miRNA processing (Jin et al., 2020; Kwon et al., 2019; Liu et al., 2018; Wang et al., 2020). In wheat, miRNAs and their targets have a complex role in controlling the growth of grains (Li et al., 2015).

Sesquiterpenes (SQs) are essential for the growth and development of plants. They function as defense agents or pheromones and were created in stressed plants. They are the most prevalent volatile secondary metabolites of plants found in nature and are part of the terpene family. They make up 28% of all terpenes found in plants. Due to their extensive involvement in the food, cosmetics, chemical, and pharmaceutical industries, they are well known. It appears that the sesquiterpene (E)-caryophyllene also acts as a defense against a bacterial disease

since the bacterial growth on the stigma of flowers without (E)-caryophyllene emission was larger than that of wild-type flowers (Huang et al., 2012). Some SQs also have a role in signal biocommunication; research disclosed a root growth stimulation caused by volatile SQ emission from adjacent fungi (Ditengou et al., 2015). SQs are well-known in the pharmaceutical industry for helping people in many ways, such as by reducing anxiety and depression (Bahi et al., 2014). Besides, current research has discovered that sesquiterpene prevents colon and skin cancer by acting as a chemopreventive agent (Picaud et al., 2006). Considering its importance in plant defense systems and commercial significance, the mechanism of sesquiterpene biosynthesis has been the focus of numerous studies. Both cosmetic and non-cosmetic items may contain the aromatic component phytol, used in many scent compounds (McCourt & Benning, 2010). However, in the medical area, phytol has demonstrated antinociceptive, antioxidant, anti-inflammatory, and antiallergenic properties (de Menezes Patrício Santos et al., 2013; Ryu et al., 2011). Recent studies have shown that phytol is an effective immunostimulant that activates innate and acquired immunity and induces long-term memory more effectively than several types of commercial adjuvants (Lim et al., 2006). Additionally, even in immune-compromised mice, phytol and its derivatives show no cumulative inflammatory or toxic consequences (Chowdhury & Ghosh, 2012).

The formation of a terpenoid usually undergoes a continuous head-to-tail addition of its building blocks, which are isoprene diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Berthelot et al., 2012; Laskovics & Poulter, 1981). First, a head-to-tail condensation of IPP and DMAPP produces geranyl diphosphate (GPP), the precursor of monoterpenoid. Then, the successive addition of IPP results in the formation of precursors of sesquiterpenoid and diterpenoid, which are farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), respectively. IPP and DMAPP are produced by two distinct processes, the predominantly cytosolic mevalonic acid (MVA) system and the plastidial methylerythritol phosphate (MEP) pathway, which both implicated the target genes *GGPS2* and *TPS13*. Figure 1 illustrates how miRNA target transcripts are involved in the terpenoid pathway. Precursors from the MVA route can be used to biosynthesize sesquiterpenoids, polyphenols, phytosterols, brassinosteroids, and triterpenoids in the cytosol, as well as terpenoids in the mitochondria. When the major isoprenoid pathway genes are overexpressed or underexpressed, this results in metabolic perturbations and changes in metabolic flux, which in turn cause feedback or feedforward signals to change the expression of upstream or downstream genes. All-trans-GGPP, produced by all-trans-GGPSs, is similar to (E, E)-FPP. It is a significant branching point for several downstream terpenoid pathways in primary and specialized

metabolism. These include the production of poly-oligoprenols, abscisic acid, strigolactones, gibberellins, plastoquinones, and diterpenoids, as well as the biosynthesis of carotenoids and the byproducts of their breakdown. Geranylgeranylated proteins are also produced. Overexpression or reduction expression of the enzyme of *GGPS2* indeed can affect the development of the plant. For instance, the phenotypes of *Ggps1* mutants show that this gene is essential for development and chlorophyll production, including seedling-lethal albinism and embryo-lethality (Ruppel et al., 2013).

Therefore, gene manipulation using miRNA or artificial miRNA has been introduced in this research. Overexpression of the selected miRNA in the transgenic plant has been proven to successfully affect the abundance of target transcripts, which are the enzymes involved in the secondary metabolite pathway. As an example, it has been reported by X. Chen (2004) as well as Tholl and Lee (2011), that even in the absence of a Class II-type 1-deoxy-D-xylose-5-phosphate synthase (DXS) enzyme, the plastidial production of monoterpene and diterpene specialized metabolites was observed at very low concentrations in *Arabidopsis* tissues. By using the metabolomic approach, the effect of miRNA overexpression on the production of secondary metabolites has been studied, specifically on sesquiterpene and phytol. Besides, the changes in the abundance of miRNAs and their target transcripts and the photosynthesis rates were also measured. Therefore, obtaining the information from this research will assist in further understanding the miRNA functions and their mechanism work on silencing the target transcript involved in the secondary metabolites pathway. Finally, this knowledge will pave the road for manipulating secondary metabolite production in plants and, ultimately, using miRNA as a tool in industry.

MATERIALS AND METHODS

Plant Materials, Plant Vectors, and Bacterial Cultures

Arabidopsis thaliana ecotype Columbia-0 seeds were used in this experiment. Wild-type plants were grown for uniformity under the standard growth condition (light intensity: 100–150 µmol/m^2/s, light period: 16 hr days/8 hr night, temperature: 22°C, relative humidity: 60%) in plant growth chambers. Plant vectors, pMDC32B-AtMIR390a-B/C, and chemically competent cells, *Escherichia coli* TOP10 and STELLAR strain (ThermoFisher Scientific, USA), and *Agrobacterium tumefaciens*, GV3101 strain, were obtained from Plant Biotechnology Lab, Universiti Kebangsaan Malaysia (UKM).

Development of amiRNAs Over-expression Constructs Using PMDC32b-AtmiR390a-b/c and pB2GW7 Vector. The amiRNAs oligonucleotides were designed by the p-sams web tool and are available on the Carrington Laboratory home page (http://p-sams.carringtonlab.org). Both oligonucleotide primers (75 bases long) had an overhang facilitating

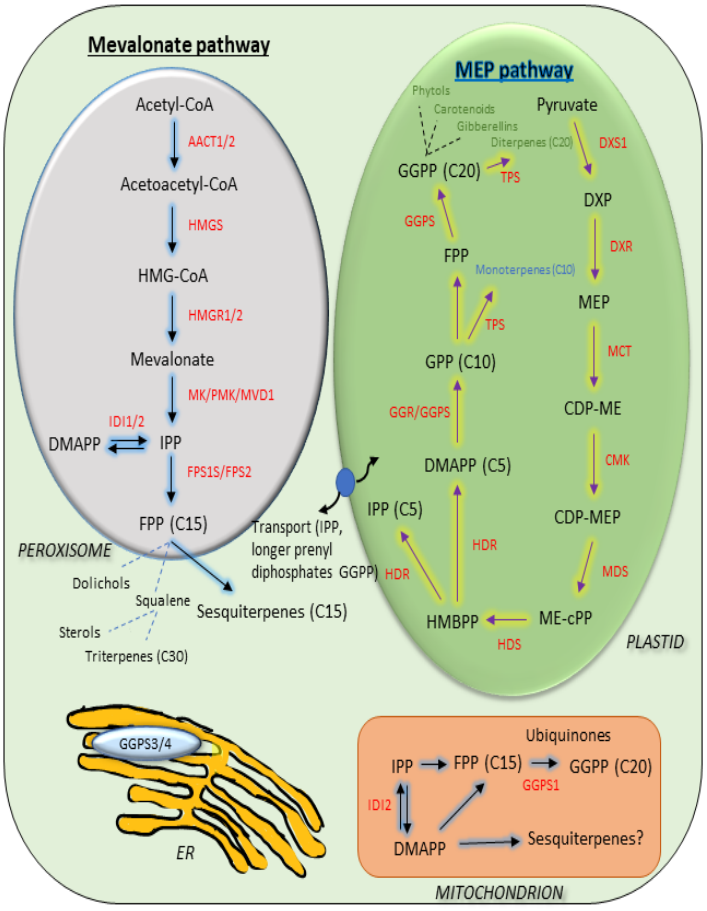

Figure 1. The subcellular structure of the terpene production pathways in *Arabidopsis*

Note. According to Sapir-Mir et al. (2008), the dashed line denotes a potential partial or complete placement of mevalonic acid pathway enzymes in peroxisomes. Involvement of the predicted target transcript *GGPS2* and *TPS13* in the terpenoid pathway (Modified from Tholl and Lee, 2011)

Note. Red terms = Enzymes: CoA = coenzyme-A; AACT1/2 = Acetyl-CoA acetyltransferase 1; HMGS = Hydroxymethylglutaryl-CoA synthase; HMGR1/2 = HMG-CoA reductase; MK = Mevalonate kinase; PMK = Phosphomevalonate kinase; MVD1 = Mevalonate-5-pyrophosphate decarboxylase; IDI1/2 = Isopentenyl-diphosphate delta-isomerase; FPS1/2 = Farnesyl pyrophosphate synthase ½; DXS1 = 1-deoxy-D-xylulose 5-phosphate synthase 1; DXR = 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MCT = Medium-chain triglyceride; CMK = 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase; MDS = 4-diphosphocytidyl-2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS = 4-hydroxy-3-methylbut-2-enyl diphosphate synthase; HDR = 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; GGR = Geranylgeranyl reductase; GGPS = Geranylgeranyl pyrophosphate synthase; TPS = Terpenoid synthase; IDI2 = Isopentenyl-diphosphate delta isomerase 2; GGPS1 = Geranylgeranyl pyrophosphate synthase 1Black terms = Molecules; HMG-CoA = Hydroxymethylglutaryl-CoA; DMAPP = Dimethylallyl pyrophosphate; IPP = Isopentenyl pyrophosphate; FPP = Farnesyl diphosphate; DXP = 1-Deoxy-D-xylulose 5-phosphate; MEP = 2-C-methyl-D-erythritol 4-phosphate (methylerythritol phosphate); CDP-ME = 4-Diphosphocytidyl-2-C-methyl-D-erythritol; CDP-MEP = 4-Diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; ME-cPP = 2-C-methyl-D-erythritol-2,4-cyclodiphosphate; HMBPP = (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate; GPP = Geranyl diphosphate; ER = Endoplasmic reticulu

ligation for cloning into the miRNA overexpression vector. The oligonucleotides pair for amiR2937 were amiR2937F: TGTAATAAGAGCTGTTGAA GGAGT CATGATGA TCACATTCG TTATCTATTTTTTGACT CCTTCACCAGCTCTTAT and amiR2937R: AATGATAA GAGCTGGTG AAGGAGTCAAAAAATAGA TAACGAATGTGATCATCATGA CTCCTTCAACAGCTCTTAT while for amiR854e, amiR854eF: TGTAGATGAG GATAGGGAGGA GGAGATGATGA TCACATTCGTT ATCTATTTTTTCTC CTCCTCCATATCCTCATC and amiR854eR: AATGGATGAGGA TATGGAGGAGGAGAAAAAATAGAT AACGAATGTGATCATCATCT CCTCCTCCCTATCCTCATC. Each amiRNA insert was synthesized by annealing the two partially complementary oligonucleotides (Carbonell et al., 2014). The pMDC32b-AtMIR390a B/c vector was joined to the annealed amiRNA inserts using the T4 DNA ligase enzyme (New England Biolabs, USA). Since this study also focuses on the co-overexpression of miRNA in *A. thaliana*, two plasmids with different markers are needed to select transgenic plants. Therefore, miR854e was cloned into the pB2GW7 vector using the Gateway Cloning technology kit from Thermo Fisher Scientific (Malaysia). The DNA fragment of interest was added with attB1/attB2 sites by polymerase chain reaction (PCR), and then the DNA fragment was cloned into pDONR221 vectors through BP Clonase procedures. PCR fragments containing the appropriate attL site were produced using a particular primer and universal primer M13. An LR Clonase recombination reaction immediately cloned the PCR fragments into the intended target vector (pB2GW7) after being gel-purified.

Developing Transgenic *A. thaliana* Plants

The GV3101 strain of *Agrobacterium* was used for the heat shock transformation method. Once the wild type of *A. thaliana* plants reached the anthesis stage, the transformation was done using the flora dip transformation method. The solution was prepared using a 100 ml Luria Betani (LB) media containing *Agrobacterium* construct colony. It was grown at 28°C until it reached an optical density (OD) of around 0.5 and was finally centrifuged to collect the cell. In 200 ml autoclaved water, 15 g sucrose (Duchefa Biochemie, Netherlands), well-grown *Agrobacterium* cell culture, and 0.025% of silwet L-77 (PhytoTech Labs, USA) were added. The plant inflorescences were then submerged for 15 to 20 s with mild agitation in the liquid *Agrobacterium* culture (Bent, 2006). The transformation was repeated twice to increase efficiency. After the plant reached maturity and was ready to be harvested, the seeds were screened through antibiotics and genomic PCR. The transgenic plant's seeds were called the T1 generation, and they were grown until the T3 generation to get a stable homozygous generation.

Growing and Sampling of *A. thaliana* Plants for Analysis

One line of T3 generation of each construct (pMDC32b_amiR2937, pB2GW7_amiR854e, and co-overexpression: pMDC32b_amiR2937 + pB2GW7_amiR854e) of the transgenic plant was selected for final analysis. The seeds were rinsed with autoclaved distilled water after being cleaned with 70% ethanol (Hamburg Chemical HmbG, Germany) for 1 min, 50% Clorox (Clorox®, Malaysia), and Tween® 20 (Duchefa Biochemie, Netherlands) for 10 min. The seeds were plated on a Murashige and Skoog (MS) media (Duchefa Biochemie, Netherlands) plate containing antibiotic (pMDC32b_amiR2937: 25 µg/ml hygromycin (Thermo Fisher Scientific, Malaysia); pB2GW7_amiR854e: 10 µg/ml BASTA (Thermo Fisher Scientific, Malaysia); and co-overexpression pMDC32b_amiR2937 + pB2GW7_amiR854e: 25 µg/ml hygromycin (Thermo Fisher Scientific, Malaysia) and 10 µg/ml BASTA [Thermo Fisher Scientific, Malaysia]). The seedlings were planted in the soil after 14 days and grown there until six weeks old. The sampling was carried out once the plants reached six weeks of age. The leaves were chopped using a sterilized scissor into a prelabeled 50 ml Rnase-free Falcon tube. Each plant's healthy leaves were collected, immediately frozen in liquid nitrogen, and kept at -80°C until analysis.

Gene Expression Analyses

RNA Extraction and First-strand cDNA Synthesis. Total RNA from each line was isolated using PureLink Plant RNA Reagent (Life Technologies, USA) according to the manufacturer's protocol. The extracted, purified RNA samples were further diluted, and NanoDrop (Thermo Fisher Scientific, USA) was used to determine the RNA's purity. Following the manufacturer's instructions, the PhotoScript II First Strand cDNA Synthesis Kit (New England Biolabs, USA) was used to synthesize first-strand cDNA. The synthesized cDNAs were stored at -80°C for further analysis.

Validation and Expression Profile using RT-qPCR. RT-qPCR was carried out to quantify the abundance and expression of the miRNAs and their target transcripts in the terpenoid pathway under *A. thaliana* inoculation using Luna® Universal qPCR Master Mix (New England Biolabs, USA). Specific primers were designed to amplify *miR* genes and their target transcript where the expected cleavage site was between the forward and reverse primers. *Act 20* gene was used as an internal control in this analysis. Table 1 lists the designed primers used in the RT-qPCR analysis.

Secondary Metabolites Profiling using SPME-GCMS Analysis

Using solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GCMS) in three biological replicates, metabolite profiling of transgenic *A. thaliana* was performed during the sixth week of growth. *Arabidopsis thaliana* leaves were harvested (approximately 1 g) in liquid nitrogen and ground into small pieces. The pulverized tissues were then immediately put into labeled solid-phase microextraction (SPME) vials to prevent the evaporation of

Table 1
List of targeted gene primers for real-time quantitative polymerase chain reaction

Targeted genes primers	Sequence (5' – 3')	Amplicon size
ACT20-F	GAAAGTCTTGCGTTCACAAAGT	104 bp
ACT20-R	TGTAATAGCGTCTAACTCCGAATC	
MIR2937-F	AAGGAGTCATGATGATCACATTCG	70 bp
MIR2937-R	GAAGAGCCAATGATAAGAGCTGG	
GGPS2-F	GGTTGCTCCTTTAGTAGCTCTTA	90 bp
GGPS2-R	ACATAAACCCAACACACACAAACA	
MIR854e-F	GGATAGGGAGGAGGAGATGATGA	103 bp
MIR854e-R	GCCTCGGCCTTTTTCATTGTAG	
TPS13-F	TGCGTTCACAAAGTTGTCTCACT	70 bp
TPS13-R	CTCCGAATCATCGATGGGAACAGAG	

volatile chemicals. Screw caps were used to close the vials, which were then heated in a water bath to 65°C for 15 min. A septum cap introduced an SPME needle to gather volatile chemicals. To absorb the volatiles, SPME fiber (100 M polydimethylsiloxane, Sigma-Aldrich, USA), which was housed inside the SPME needle, was injected into the vials (Câmara et al., 2006). The collecting vial's SPME fiber equilibrium time was 30 min. at 55 to 60°C. The non-polar column was a diameter of HP-5MS (Agilent, USA, 30 m x 0.25 mm x 0.25 m). A splitless injection was set at 50°C held for 3 min, increased to 100°C at a rate of 20°C/min, and held at 250°C for 3 min. A GC-MS chromatogram was created using the results of a search of the NIST/EPA/NIH spectrum library (version 11) to locate the peaks.

Total Chlorophyll Content Analysis using SPAD-METER 502

When the plant reaches the age of 6 weeks, the chlorophyll content in the leaves is read using a SPAD-METER 502 (DS Technology, Malaysia). Readings were taken on 12 individual *A. thaliana* plants from the same line. The reading method was taken by reading two leaves on each plant three times, and the average reading was taken.

Total Chlorophyll Content Analysis Using Acetone Extraction Method

Chlorophyll extraction was performed following a protocol developed by (Liang et al., 2017). Two leaves from the previous analysis were cut into a circle using a cork borer. For at least 24 hr, the leaves were incubated in a 2 ml Eppendorf tube containing 1 ml of an 80% acetone solution (1Malaysia Bio Lab, Malaysia). The solution was then centrifuged at 1,957 x *g* for 5 min. At wavelengths of 646 and 663 nm (A_{646} and A_{663}), the absorbance of the supernatant was determined in this investigation. Samples with absorbance greater than 1 had their concentrations of 80% acetone cut in half before being re-analyzed. Following Arnon's equation, the amount of chlorophyll was calculated as follows:

Chlorophyll *a* (µg/ml) = 12.7 (A_{663}) – 2.69 (A_{645})

Chlorophyll *b* (µg/ml) = 22.9 (A_{645}) – 4.68 (A_{663})

Total chlorophyll (µg/ml) = 20.2 (A_{645}) + 8.02 (A_{663})

The area of the leaves was kept constant, and chlorophyll content and the total chlorophyll content per leaf area were expressed as ng/mm^2.

Gaseous Exchange

Gas exchange measurements were conducted using the Li-6800 portable gas exchange device (LI-COR, Malaysia). Photosynthetic rate readings were read on six plants for each line of transgenic and wild-type plants. All measurement data were collected between 8:00 a.m. and 12:00 p.m. Light saturation was set at 120 µmol photons/m^2/s, and carbon dioxide (CO_2) surrounding the leaves was set at 400 µmol/mol to induce photosynthesis rates. In addition, to maximize the stomatal aperture, the blue light was set to 10% of the active photosynthetic photon flux density. Measurements were made when the leaf temperature was ~25°C.

Statistical Analyses

A *t*-test was performed for statistical analysis to determine significant differences among the samples. Differences were taken as significant when the *P*-value was < 0.05.

RESULTS AND DISCUSSION

Selection and Confirmation of Transgenic Plants through Genomic PCR

Genomic PCR analysis was done when the plant had grown true leaves. The genomic DNA was extracted following the protocol prepared by (Kasajima et al., 2004). As shown in Figures 2, 3, and 4, the tested plants showed the correct amplicon size for the inserts: miR2937 insert with a band of 366 bp, and miR854e insert with a band of 312 bp. Meanwhile, the transgenic plant co-overexpressed both constructs and showed the correct amplicon size for both inserts, 312 bp and 366 bp. This step was crucial to ensure that the desired insert was copied into the genome of the transgenic plants.

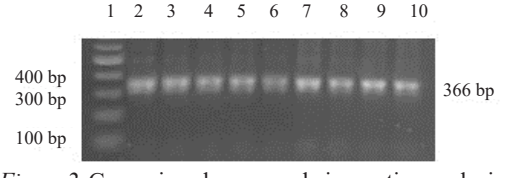

Figure 2. Genomic polymerase chain reaction analysis of transgenic plant harboring construct pMDC32b_ amiR2937 with the size of amplification 366 bp

Note. Lane 1 = 100 bp ladder (Invitrogen, USA); Lanes 2-10 = Transgenic plant harboring construct pMDC32b_amiR2937

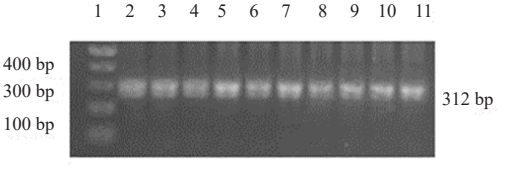

Figure 3. Genomic polymerase chain reaction analysis of transgenic plant harboring constructs pB2GW7_ miR854e with a size of amplification 312 bp

Note. Lane 1 = 100 bp ladder (Invitrogen, USA); Lanes 2-11 = Transgenic plant harboring construct pB2GW7_ miR854e

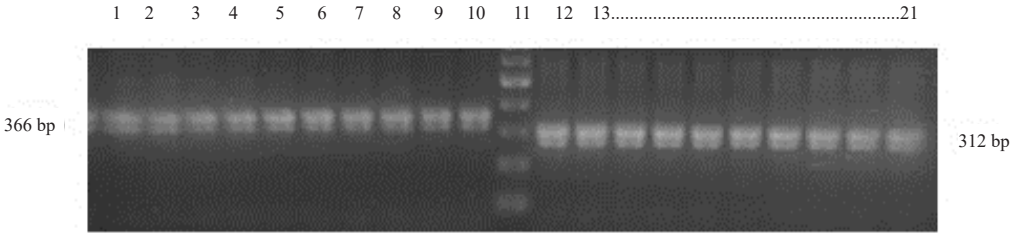

Figure 4. Genomic polymerase chain reaction analysis of transgenic plant harboring both constructs pMDC32b_amiR2937 and pB2GW7_amiR854e with a size of amplification 366 bp and 312 bp, respectively

Note. Lane 11 = 100 bp ladder (Invitrogen, USA); Lanes 1-10 and lanes 12-21 = Transgenic plant harboring both constructs pMDC32b_amiR2937 and pB2GW7_amiR854e, respectively

Expression Profiles of miRNAs and Their Target Transcripts by RT-qPCR

Quantitative expression analysis of pMDC32b_amiR2937 and pB2GW7_amiR854e and their target transcript, *GGPS2* and *TPS13*, were investigated in leaves of the *Arabidopsis* compared to the wild type. The specified miRNA's expression and target transcripts were experimentally validated using an RT-qPCR. The expression profile was shown in Figure 5, whereas pMDC32b_amiR2937 was upregulated by 0.89-fold. Meanwhile, co-overexpression of amiR2937 + amiR854e was significantly upregulated by 1.88-fold. Its target transcript, *GGPS2*, was significantly downregulated in both transgenic lines by 0.89- and 0.92-folds, respectively. The decrease in the accumulation of *GGPS2* mRNA in the transgenic plants overexpressing amiR2937 shows that amiR2937 has successfully suppressed the target (*GGPS2*) as compared to the wild type, where the abundance of amiR2937 and *GGPS2* was unaffected. The simplest outcome of the condensation reaction between DMAPP and IPP is C10 geranyl diphosphate (GPP), the precursor of monoterpenoids, which is controlled by GPPS present in plastids (Beck et al., 2013; Chen et al., 2015). An RT-qPCR study revealed that, among 17 different miRNAs, miR2937 showed a change in abundance in the pollen as compared to the leaves and had targeted the activator of spomin: luc2 (ASML2), which activates the promoter of sugar-inducible genes (Grant-Downton et al., 2009).

In Figure 6, the relative gene expression of amiR854e was significantly increased in both transgenic plants compared to the wild type. There was an upregulation by 1.81-fold in pB2GW7_amiR854e and 2.52-fold in amiR2937 + amiR854e. The transgenic lines that overexpress amiR854e were predicted to target *TPS13* genes. Accordingly, the result showed a downregulation of the target transcript by 0.84-fold in pB2GW7_amiR854e and 0.87-fold in amiR2937 + amiR854e. There is no such study where *TPS13* has yet been reported as target transcripts of miR854e. It is the first study to report that amiR854e overexpression caused the target transcript, *TPS13*, to be downregulated. The target, *TPS13*, was a type-a *Arabidopsis* TPS and a Class I protein. This Class I protein lacks

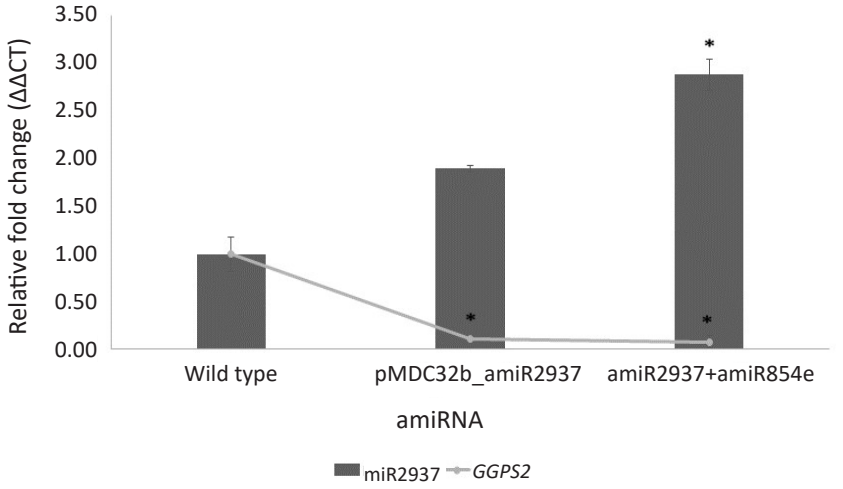

Figure 5. Graphical representation of relative gene expression of amiR2937 and *GGPS2* target transcripts in control and transgenic plants

Note. Asterisk(*) indicates the significant difference in gene expression towards control (*t*-test, $P < 0.05$)

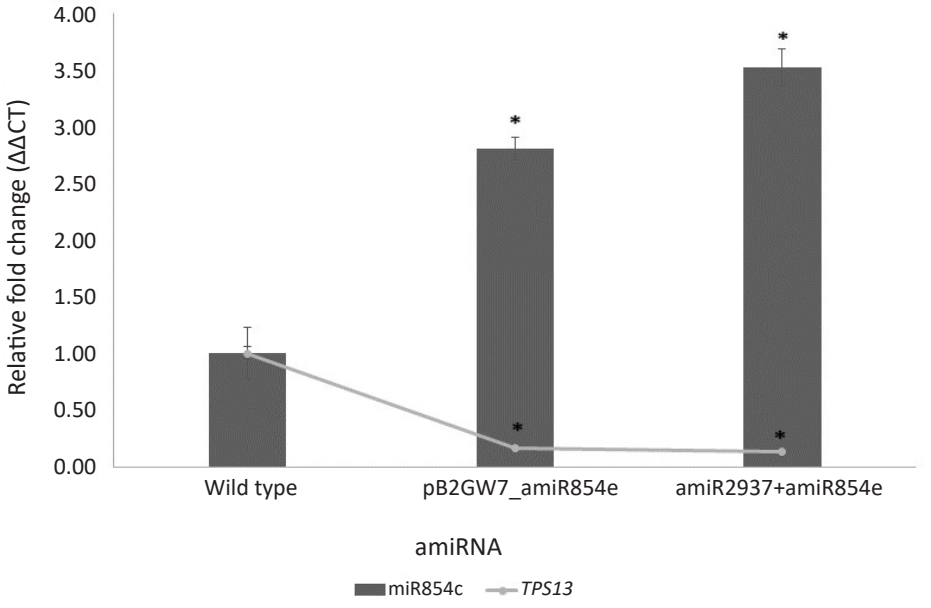

Figure 6. Graphical representation of relative gene expression of amiR854e and *TPS13* target transcripts in control and transgenic plants

Note. Asterisk(*) indicates the significant difference in gene expression towards the control (*t*-test, $P < 0.05$)

a y-domain and carries a non-functional N-terminal domain (Tholl & Lee, 2011). According to Tholl and Lee (2011), *TPS13* was included in four type-a *Arabidopsis* TPS genes (*TPS11*, *TPS12*, *TPS13*, and *TPS21*). Sesquiterpene synthases are proteins encoded by these TPS genes, which lack plastidial transit peptides.

Both *TPS12* and *TPS13* enzymes catalyzed the synthesis of the minor products (E)-nerolidol and (Z)-y-bisabolene, which are both derivatives of bisabolol (Ro et al., 2006). It was discovered that the animal and plant realms possess the miR854 gene. Previous research identified that miR854 in *A. thaliana* had homologs in animal and human genomes by one nucleotide difference. The miR894 targeted the UBP1 protein, which was involved in transcription (Arteaga-Vázquez et al., 2006). Besides, in *A. thaliana*, family miR854 consists of another four family members beginning with miR854a, miR854b, miR854c, and miR854d (Kozomara & Griffiths-Jones, 2014).

Terpenoid Profiling in Transgenic *A. thaliana* by SPME-GCMS Analysis

SPME-GCMS analysis was carried out to reveal the terpenoid content in transgenic *A. thaliana* compared to the wild type. The GC-MS result in Figure 7 above shows that the amount of phytol (a diterpenoid) in transgenic plants overexpressing pMDC32b_amiR2937 was reduced by 1.86%. The reduction was not significant, which may be due to the stability of the transgene and its ability to be expressed in plants. Many factors can affect how transgene expression varies between individuals. For instance,

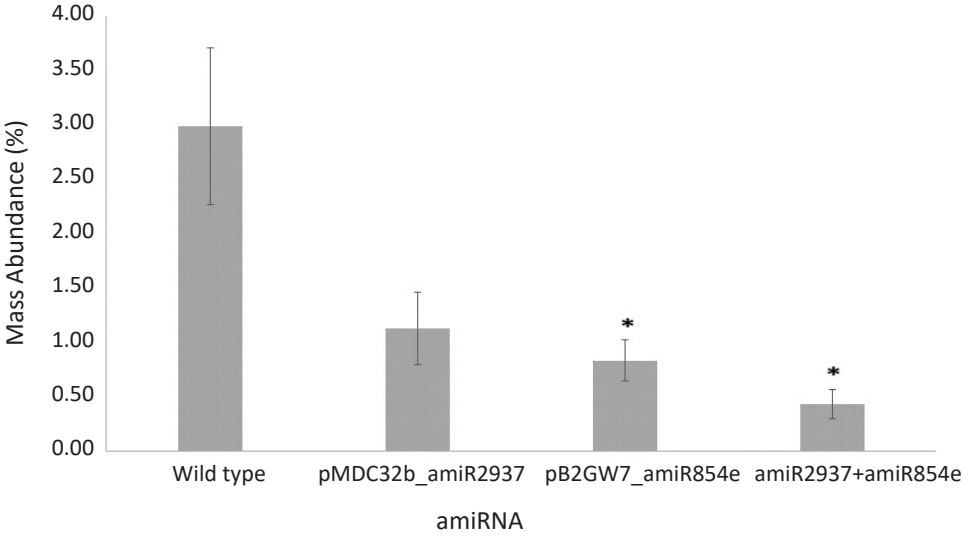

Figure 7. Graphical representation of changes in mass abundance of phytol in transgenic plants as compared to the wild-type

Note. Asterisk(*) indicates the significant difference in the abundance of phytol towards the control (*t*-test, $P < 0.05$)

promoters can influence the degree and diversity of transgene expression in different transformants (De Bolle et al., 2012). On the other hand, the amount of phytol in both transgenic plants harboring construct pB2GW7_amiR854e and amiR2937 + amiR854e were significantly downregulated by 2.16 and 2.56%, respectively.

Interestingly, based on the result, the abundance of phytol was greatly reduced in transgenic plants harboring amiR854e, even though the level of phytol is dependent on the level of *GGPP* transcript, which is regulated by amiR2937. According to Tholl (2015), TPS enzymes vary in their substrate specificity from using only a single prenyl diphosphate substrate to converting two or more substrates (e.g., GPP and FPP) *in vitro*. However, it depended on their subcellular localization. TPS enzymes located in plastids generally produce monoterpenes or diterpenes from the predominantly plastidial pools of GPP and GGPP, respectively. Meanwhile, TPSs in the cytosol primarily convert FPP to sesquiterpenes (or squalene in the biosynthesis of C_{30} terpenes) (Tholl, 2015).

The trans-β-ionone was a sesquiterpenoid compound with an increasing pattern compared to the wild-type plant. This compound is a precursor of carotenoids. This compound is of great biological value as it possesses anti-cancerous, antimicrobial, and anti-mutagenetic activities (Kang et al., 2013). From Figure 8, there was an up-regulation of the beta-ionone compound in all transgenic plants, and it was highest in transgenic plants overexpressing both

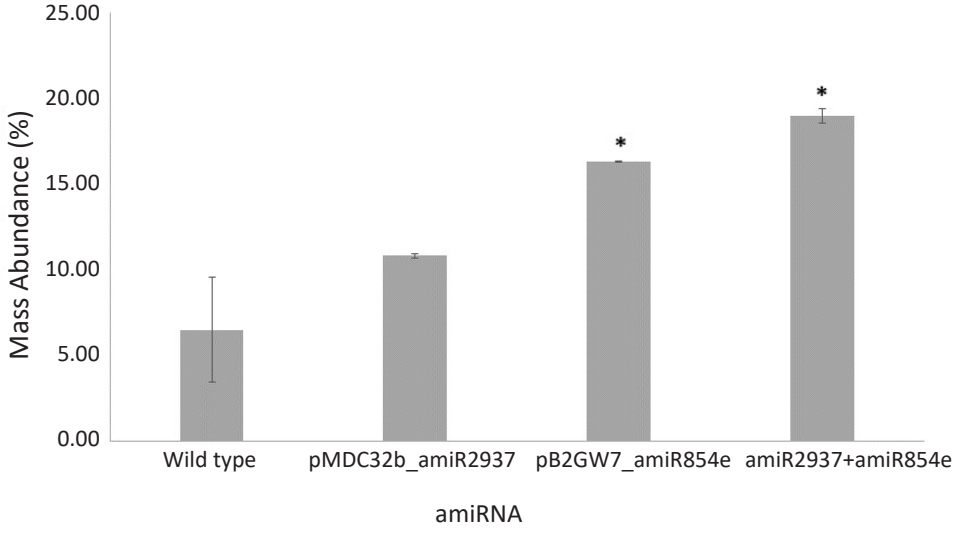

Figure 8. Graphical representation of changes in mass abundance of trans-beta-ionone in transgenic plants as compared to the wild-type

Note. Asterisk(*) indicates the significant difference in the abundance of trans-beta-ionone towards the control (*t*-test, $P < 0.05$)

constructs, amiR2937 + amiR854e, which was 12.49%. Based on the graph, trans-beta-ionone was slightly upregulated in the mutant, pMDC32b_amiR2937, by 4.3%. Meanwhile, in transgenic plants harboring construct pB2GW7_amiR854e, trans-beta-ionone was upregulated by 9.82%. From the analysis, transgenic plants that overexpressed amiR854e showed more upregulated trans-beta-ionone compounds compared to amiR2937. To our knowledge, *TPS13* was involved downstream of the MEP pathway of terpenoid biosynthesis. Theotherically, overexpression of miRNA in the plant would downregulate the production of the adjacent secondary metabolites. For example, the downregulation of sesquiterpene levels in *Pogostemon cablin* and *A. thaliana* is caused by the overexpression of miR-156.

Similarly, the overexpression of miR-393 alters glucosinolate and camalexin levels by disrupting the auxin signaling system (Yu et al., 2015). Therefore, the overexpression of miRNA in the plant definitely can change the production of the metabolites. However, whether the amount is up- or down-regulated depends on the pathway, which is still unknown. According to Tholl (2015), combinatorial mutations in downstream terpene synthases and prenyl diphosphate synthase are one method to increase pathway productivity. For instance, the level of the levopimaradiene product increased more than 2,000-fold when a route variation of a GGPPS and a terpene synthase that produces a levopimaradiene diterpene precursor was expressed in prokaryotes (Leonard et al., 2010).

Quantifying the Amount of Chlorophyll Content in *A. thaliana* Transgenic Plants by Using SPAD-METER 502 Reading

The relative soil plant analysis development (SPAD) values generated by the SPAD-502 meter are proportionate to the amount of chlorophyll in the leaf. In addition, the amount of chlorophyll in leaves is a general measure of chloroplast proliferation, photosynthetic ability, and leaf nitrogen content (Ling et al., 2011). This analysis aims to study the effect of miRNA overexpression in transgenic lines of *A. thaliana* on chlorophyll content. It should be noted that altering growing conditions, such as those that can cause a redistribution of chloroplasts inside mesophyll cells, can impact SPAD meter readings (Nauš et al., 2010). As a result, it is advised to refrain from comparing tests performed at different times and to always include an internal control as a point of comparison in every experiment. However, as the plants being studied are grown under the common, strict, and controlled circumstances employed here, the results should be accurate. The measuring area of the meter, which is 2 mm x 3 mm, is another thing to keep in mind.

However, by taking numerous measurements using various sections of the same leaf, this issue might be at least partially alleviated. From Figure 9, the SPAD value reading and chlorophyll content in pMDC32b_amiR2937 was significantly decreased by 1.644 nmol chl/cm^2. This transgenic plant overexpressed amiR2937, which was predicted to target *GGPS2*.

Theoretically, when the abundance of the miRNA increases, the *GGPS2* is predicted to decrease. As the *GGPS2* enzyme was involved in the MEP pathway and produced the product of chlorophyll, this result showed that the amount of chlorophyll content was decreased. It had been reported by Beck et al. (2013), in *A. thaliana*, there were twelve members of the GGPS reported being positioned in a variety of subcellular compartments, including the mitochondria, endoplasmic reticulum (ER), or chloroplast. The enzymatic product of trans-geranylgeranyl diphosphate synthase, (E,E,E)-GGPP was an important branch point enzyme because it served as a key precursor for a wide range of primary and specialized isoprenoid compounds, such as carotenoids and carotenoid breakdown products. Besides, for pB2GW7_amiR854e, the SPAD value was significantly decreased by 1.203 nmol chl/cm^2. These transgenic lines had overexpressed miR854e, which was predicted to target the *TPS13* gene. Meanwhile, in the transgenic lines that harbored both constructs, amiR2937 amiR854e, the SPAD value reading was decreased by 1.139 nmol chl/cm^2.

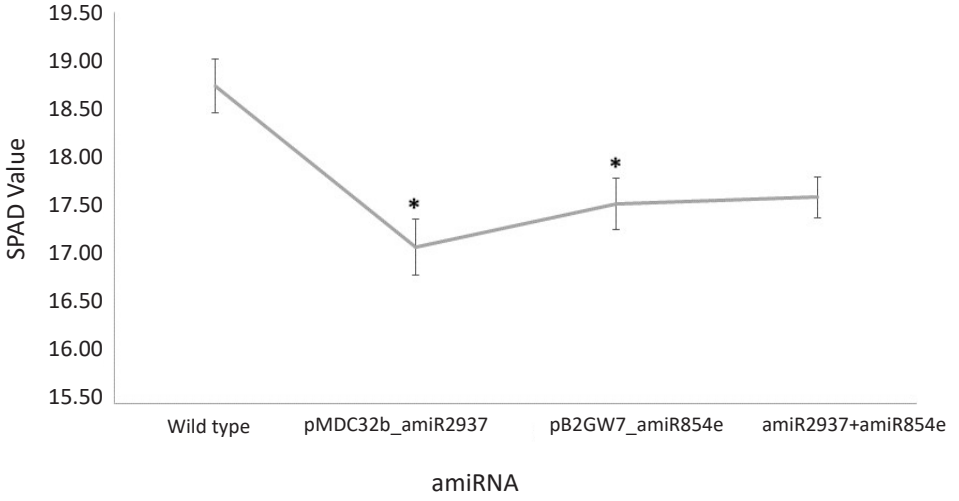

Figure 9. Graphical representation of soil plant analysis development (SPAD) value and chlorophyll content per leaf area in the transgenic plants compared to the wild-type

Note. Asterisk(*) indicates the significant difference in the chlorophyll content towards the control (*t*-test, $P < 0.05$)

Chlorophyll *a*/*b* and Total Chlorophyll Content by Acetone Extraction

This study analyzed the differences in chlorophyll *a*/*b* and total chlorophyll content between the transgenic plants and the wild type. The main photosynthetic pigment is chlorophyll *a* (chl *a*), while chlorophyll *b* (chl *b*) is merely an auxiliary pigment. Therefore, there was a huge difference between the content of chl *a* and chl *b* in

Figure 10. Chl *a* present in all plants, algae, bacteria, cyanobacteria, and phototrophs, but chl *b* only exists in green algae and plants. Figure 10 shows that all transgenic plants had much lower chl *a* and *b* levels than the wild-type plants. There was down-regulation of chl *a* in pMDC32b_amiR2937, pB2GW7_amiR854e, and amiR2937 + amiR854e by 2.661, 0.99, and 2.133 µg/ml, respectively. Meanwhile, chl *b* was downregulated by 0.770, 0.394, and 0.643 µg/ml.

Total chlorophyll content per leaf area in Figure 11 showed a decreasing pattern in all transgenic lines and was highest in pMDC32b_amiR2937 by 244.09 ng/mm^2. This result was consistent with the SPAD value above, showing a decreasing pattern in the same transgenic plants. Meanwhile, transgenic plants that harbor construct pB2GW7_amiR854e indicated the lowest total chlorophyll content by 81.48 ng/mm^2. Interestingly, total chlorophyll content in transgenic plants harboring double miRNA construct, amiR2937 + amiR854e, also decreased but was higher than pB2GW7_amiR854e by 192.37 ng/mm^2. Overall, the result indicated that amiR2937 may influence the total chlorophyll in the transgenic plant compared to the wild type. Moreover, the SPAD value result in Figure 9 also revealed that the transgenic plant harboring miR2937 construct has the lowest chlorophyll content per leaf area. As the target transcript, *GGPS2* was indirectly involved in the production of phytol in the terpenoid pathway; the overexpression of amiR2937 may affect the total chlorophyll production in the transgenic plant.

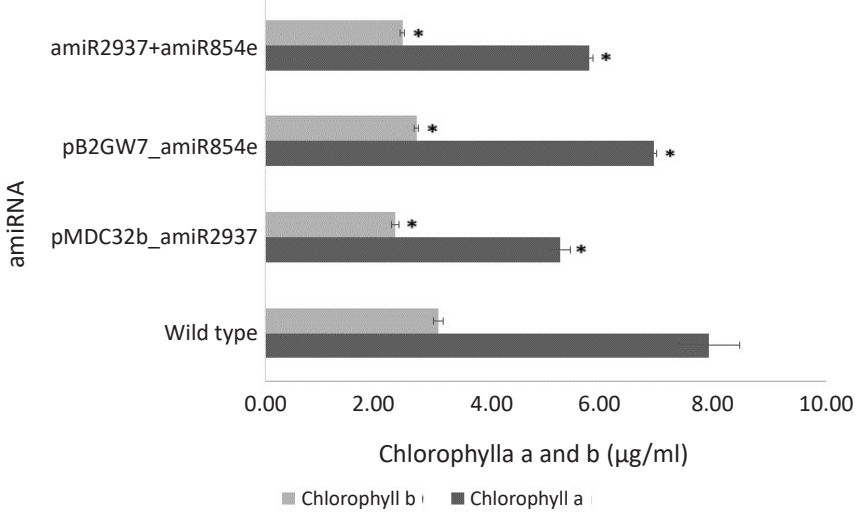

Figure 10. Graphical representation of chlorophyll *a* and *b* in transgenic plants compared to the wild type
Note. Asterisk (*) indicates the significant difference in the chlorophyll a/b towards the control (*t*-test, $P < 0.05$)

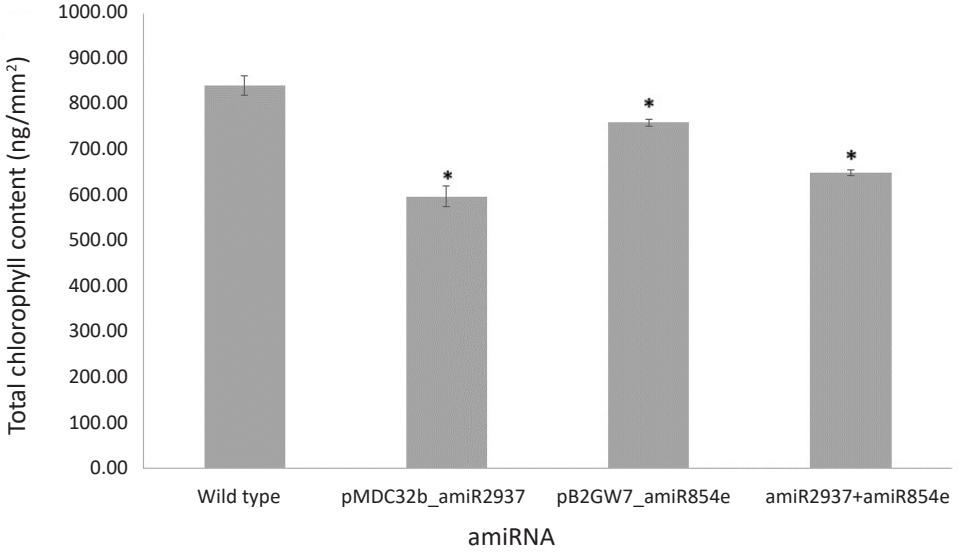

Figure 11. Graphical representation of total chlorophyll content in the transgenic plants compared to the wild-type

Note. Asterisk(*) indicates the significant difference in the total chlorophyll content towards the control (*t*-test, $P < 0.05$)

Effect of Phytol Content Degradation in *A. thaliana* Transgenic Plants Towards Gas Exchange Performances

As photosynthesis in plants occurs at its maximum during those hours, gaseous exchange analysis was carried out between 8 a.m. and 12 p.m. The rate of photosynthesis could be influenced by stomatal conductance, light intensity, and carbon dioxide concentration in the leaves (Feng et al., 2019). The plants were grown under constant conditions, light intensities, controlled surrounding temperature, relative humidity, and water supply throughout the analysis. These variables can affect the rate of photosynthesis in plants because diverse processes associated with growth and development depend on the interaction of intracellular organelles. In addition, the primary location for photosynthesis, the chloroplast, is extremely susceptible to a variety of adverse situations, including salinity, drought, extremely high or low temperatures, flooding, fluctuating light levels, and UV radiation (Saravanavel et al., 2011). Plus, phytol, a chlorophyll component, can surely influence gas exchange performances in plants.

Consistent with the degradation of phytol content, all transgenic plants showed a decreasing photosynthesis rate pattern, as shown in Figure 12. The gas exchange in pMDC32b_amiR2937, pB2GW7_amiR854e, and amiR2937 + amiR854e was slightly decreasing by 1.262, 1.364, and 1.144 µmol/m²/s, respectively. The photosynthesis rate was also consistent with the total chlorophyll content and

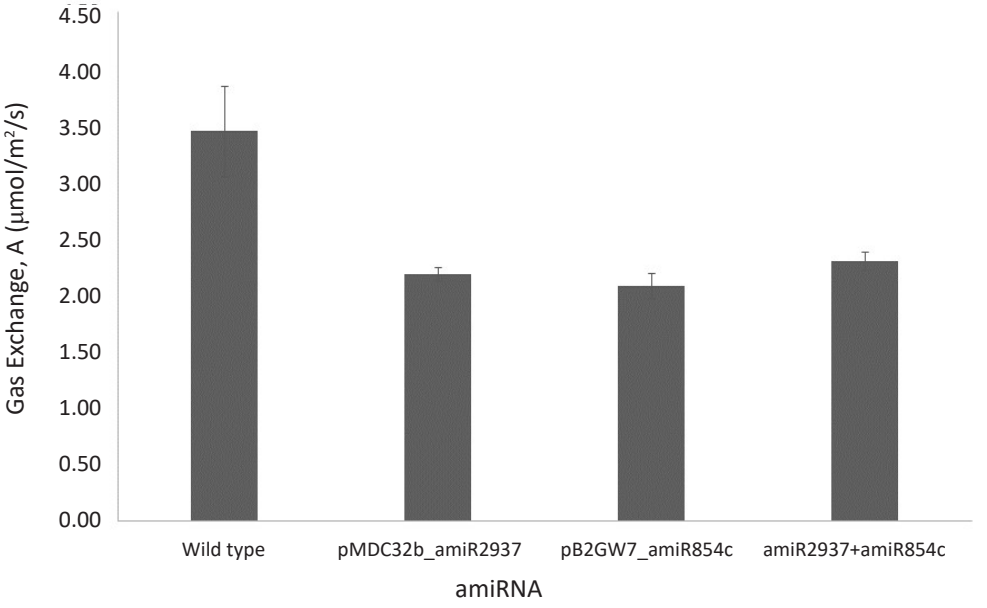

Figure 12. Graphical representation of gaseous exchange in transgenic plants compared to the wild-type
Note. Asterisk(*) indicates the significant difference in gaseous exchange towards the control (*t*-test, $P < 0.05$)

SPAD value result, which showed a decreasing pattern in all transgenic plants. Besides chlorophyll content, leaf stomatal conductance is essential for CO_2 uptake and desiccation prevention. Reduced leaf turgor, atmospheric vapor pressure, and chemical signals created by roots are the main causes of stomata closure in response to drought and salinity stress. Due to stomatal transport restrictions for gases, salt, and drought adversely influence photosynthesis and mesophyll metabolism (Chaves et al., 2009; Parida et al., 2005). Therefore, throughout the analysis, the stomata conductance was maintained and stable to minimize the technical error.

CONCLUSION

This study demonstrates that overexpression of amiR2937 and amiR854e in the transgenic plant of *A. thaliana* had successfully suppressed their target transcript, *GGPS2* and *TPS13*. All transgenic plants drastically reduced the target transcript levels compared to the wild type. The change in the abundance of phytol, which was the component of chlorophyll, is analyzed through GCMS analysis. Consistent with the downregulation of the target transcripts, the abundance of phytol in all transgenic plants showed a decreasing pattern compared to the wild type. Meanwhile, trans-β-ionone, a sesquiterpenoid compound, had increased abundance compared to the wild-type plant. The overexpression

of amiR854e revealed that it could affect trans-beta-ionone production when the amount was significantly increased in single and co-overexpression plants. The SPAD value analysis was consistent with the phytol metabolite result, which showed a decreasing pattern in the transgenic plant. SPAD value indicates chlorophyll content in the plant, and the overexpression of both miRNA, amiR2937 and amiR854e, had decreased its amount. Meanwhile, the photosynthetic activity and total chlorophyll content in the transgenic plants also decreased compared to the wild type. Moreover, both target transcripts, *GGPS2* and *TPS13*, were directly involved in the production of phytol (diterpenoid) in plants, and the overexpression of these miRNAs can affect its production. Based on this study, the expression of the downstream miRNA amiR854e has shown a significant change pattern in all the analyses carried out because the position of the downstream target transcript is more specific.

Next, this study suggests studying the functionality of other miRNAs involved in downstream pathways, such as miR5631, miR835-5p, and miR393a-5p. Furthermore, there is no other report on the functionality of the miRNA that has been done, so this research will pave the way to understanding the role of the miRNA in the terpenoid biosynthesis pathway. Many more secondary metabolites with high value in the market can be explored. Besides, the *Arabidopsis* genome contains a gene family of 11 predicted GGPSs (GGPS1-11) (Lange & Ghassemian, 2003) that were in different organelles: plastids, cytosol, endoplasmic reticulum, peroxisome, and mitochondria (Tholl & Lee, 2011). Using different algorithms, analysis of putative targeting sequences suggested five other *GGPS* isoforms to be plastidial proteins (*GGPS2*, 5, 10, 11). As the *GGPS2* enzymes were in the plastid, it is suggested that the other plastidial GGPS isoforms be studied as well. Moreover, an in-depth study of the GGPS function should be carried out by allocating the GGPP precursors and controlling the metabolic flux to distinguish terpene biosynthetic pathways.

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