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Molecular Regulation of the Metabolic Pathways of the Medicinal Plants: *Phyla dulcis*

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Abstract

***Phyla* (*Lippia*) *dulcis* contains hernundulcin sesquiterpene zero-caloric sweetener that is about a thousand times sweeter than sucrose, and also bitter constituents including camphor and limonene. There is yet no simple method to remove the undesirable constituents. The yield of sweetener hernundulcin is very low, and there is no simple method to maximize its composition. The aim of the project was to characterize the mRNA targets that regulate the primary and terpenoid metabolic enzymes of *P. dulcis*. Restriction fragment differential display polymerase chain reaction of *P. dulcis* glutamate dehydrogenase-synthesized RNA showed that many mRNAs encoding *β*-caryophyllene, (+)-epi-*α*-bisabolol, bicyclogermacrene, bifunctional sesquiterpene, and geraniol synthases shared sequence homologies with ribulose-1,5-bisphophatase carboxylase, granule-bound starch synthase, pyruvate kinase, glucose-6-phosphate dehydrogenase, and phosphoenol pyruvate carboxylase. Sequence similarities between mRNAs encoding primary metabolic enzymes and terpene synthases suggested that photosynthesis could regulate terpenoid metabolism in order to increase the yield of sweetener hernundulcin.**

Keywords

Hernundulcin Sweetener, Primary Metabolism, Terpene Synthase mRNA, Glutamate Dehydrogenase

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1. Introduction

Phyla (*Lippia*) *dulcis* (Verbenaceae) is a Central American plant used traditionally by Aztac peoples as herbal sweetener [1]. Herbal ingredients in dietary supplements for control of diabetes and obesity are gaining popularity and have a huge market [2]. However, more scientific studies on these medicinal plants are needed in order to bring them to commercial products. One of the predispositions to obesity is the intake of high caloric foods. Therefore more research was focused on the chemistry of zero-calorie sugar substitutes. There are over ~100 medicinal plant-derived sweet compounds of 20 major structural types that have been reported, and are isolated from more than 25 different families of green plants [1]. The Central American plant *Phyla dulcis* has two sesquiterpene sweetener constituents: hernandulcin and 4*β*-hydroxyhernandulcin which are 1000 times sweeter than sucrose [3]. As such, they have potential for use as natural low calorie sweeteners in the dietary management of diabetes/obesity. However, other compounds identified in *Phyla* extracts are undesirable and include camphor, limonene, terpineol, *α*-pinene, *α*-copaene, trans-caryophyllene, *δ*-cadinene, and *α*-bisabolol [4]. Efforts are in progress to remove the camphor from leaf extract through hydro-distillation [4], supercritical fluid extraction technology [5]; microbial degradation of the camphor [6]; hernandulcin production in yeast [7] [8]; and/or production of hernandulcin by hairy root/shoot culture of *P. dulcis* [9]. *Phyla* species are characterized by variability in the chemical compositions of their monoterpenes, and sesquiterpenes depending on the origin of plant materials, and the stage of maturity of the plant part selected for analysis. Various metabolic variants (chemotypes) of *Lippia alba* have been identified [4]. But the molecular regulation that conferred the biochemical characteristics on the chemotypes was not studied. *Phyla* species abound in terpene and sesquiterpene synthases [7] [10] [11]. Therefore it is anticipated that some of the regulatory targets would be associated with the mRNAs encoding the monoterpene and sesquiterpene synthases. Earlier results demonstrated the successes in the utilization of some environmental conditions (mineral salts, and nucleotide solutions) to alter the yields of fatty acids, resveratrol, amino acids, cellulose, proteins, and biomass feedstock metabolic pathways of crops including sweetpotato, soybean, peanut, and cowpea [12]-[14]. The mechanism is that the stoichiometric mineral ion, and nucleotide mixes induced the glutamate dehydrogenase (GDH) to isomerize and to synthesize some RNAs which silence mRNAs homologous to them [12]-[17]. The research approach [13] has explained many hitherto inexplicable biological phenomena including the production of arachin-free peanut [14], metabolic detoxification of xenobiotics in plants [15], doubling of peanut yield [16], and of ultra-high resveratrol peanut [17]. GDH is the target site of the action of nucleophiles including nucleotides, mineral ions etc. [18] [19]. Such plant-based systems research approaches have not been applied to *Phyla* species. With the perfection of the genetic basis of yeast fermentation production of sesquiterpenes [8] accompanied by the detailed analytical chemistry of terpenes [6] [7] [10], it is logical to focus research attention on potential agricultural technologies for production of high-hernandulcin *P. dulcis* metabolic variants. *Phyla* sweetener industry generates about $1.5 billion [2] [20]. Wherefore, the proof of the biochemical protocols for the GDH-based plants system research concept is the demonstration of the RNA synthetic activity [21] of *P. dulcis* GDH and characterization of the metabolic functions of the RNA as presented hereunder.

2. Materials and Methods

2.1. Cultivation of Medicinal Plants *Phyla dulcis*

Stem cuttings from *P. dulcis* (Trev.) Mold. (Verbenaceae) growing in a peat moss potting medium in plastic containers in a greenhouse were planted on raised beds in March-April, 2013 in the medicinal plants garden of Prairie View A & M University Research Farm, and watered by drip irrigation as necessary without fertilizer treatment. The soil at the site is characterized as a fine-loamy, siliceous, thermic Typic Paleudalf. The weather conditions throughout the *Phyla* establishment were monitored at the USDA-NRCS Scan Station on the Prairie View A & M University farm a few meters from the *Phyla* plot. The *Phyla* established rootings quickly and commenced good vegetative growth. In late April, green and young leaves were harvested from many healthy plant stands, frozen in dry ice and transported to storage in −80˚C freezer. The *P. dulcis* continued to grow in the plots, and in June-July the intense summer light and heat of Texas induced the purple coloration of the older leaves.

2.2. Purification and Assay of GDH

GDH was extracted with Tris-HCl buffer solution containing RNAse A and DNase 1, and purified by electro-

phoresis as described before [21] [22] from *P. dulcis* green leaves harvested from the medicinal plants garden. RNA synthetic activity of GDH isoenzymes [12] was assayed in combined deamination and amination substrate solutions of 0.1 M Tris-HCl buffer solution (pH 8.0) containing the four NTPs (0.6 mM each), $CaCl_2$ (3.5 mM), L-glu (3.23 μM), NAD^+ (0.375 μM), NH_4Cl (0.875 mM), α-ketoglutarate (10.0 mM), NADH (0.225 mM), 5 Units RNase inhibitor, 1 Unit DNase 1, and 5 μg of actinomycin D. Reaction was started by adding 0.2 mL of whole gel-eluted GDH charge isomers containing 3 - 6 μg protein per mL. Final volume of the reaction was brought to 0.4 mL with 0.1 M Tris-HCl buffer pH 8.0. Reactions were incubated at 16˚C overnight and stopped by phenol-chloroform (pH 5.5) extraction of the enzyme. RNA was precipitated with ethanol, and dissolved in minimum volume of molecular biology quality water. RNA yield and quality were determined by photometry and by agarose gel electrophoresis. Assays were carried out in duplicate to verify the reproducibility of the results.

2.3. Complementary DNA Synthesis, Cloning and Characterization

cDNAs were synthesized with 2 μg of each product RNA synthesized by the whole gel-eluted GDH charge isomers using random hexamer primer. Restriction fragment PCR amplification; adapter ligation; sequencing gel fractionation; and purification of cDNA fragments [12] [13] were conducted according to the methods of Display Systems Biotech, Vista, CA, USA. Selected cDNA fragments were subcloned into pCR4-TOPO vector and transformed into TOP10 One Shot Chemically Competent *Escherichia coli* (Invitrogen, Carlsbad, CA), followed by overnight growth on selective plates. Up to ten positive transformant colonies were picked per plate and cultured overnight in LB medium containing 50 μg/mL of kanamycin. Plasmid DNA was purified with a plasmid kit (QiaGen mini kit, Madison, WI). The insert cDNA was sequenced with T3 and T7 primers by Functional Biosciences, Inc. (Madison, WI, USA).

To characterize the GDH-synthesized RNAs, the cDNA sequences were used as queries to search the NCBI nucleotide-nucleotide (excluding ESTs) BLAST (blastn), and non-redundant protein translation (blastx) databases for *Phyla dulcis* and related taxids. Complementary DNAs that displayed the highest alignment scores with mRNAs encoding the enzymes of primary and natural products (secondary) metabolism were selected.

3. Results and Discussion

3.1. Cultivation of *P. dulcis* in Texas, USA

Phyla dulcis, prostate perennial medicinal herb with many branches, originally a South American sweet herb has been established in the medicinal plants garden of the University, Waller County, Texas, USA. The weather conditions for March and April 2013 (**Table 1**) showed that rainfall decreased but temperatures increased during the period of the experiment. The weather conditions are important for reporting experimental conditions and for future field plot establishment of the species. Under the intensive light, heat and humidity conditions of Texas summer, the older leaves turned purple (**Figure 1**) suggesting that the photosynthetic pathways could be subject to environmental regulation. There is no literature record on the sustainable cultivation of the species in the USA, therefore environmental research is in progress for identifying best management strategies (fertilization, plant protection, irrigation etc.).

3.2. *Phyla dulcis* GDH Activity

Rotofor isoelectric focusing (IEF) of *P. dulcis* GDH extracts gave a single horizontal row of isoenzymes covering from Rotofor chambers (fractions) 4 to 12 on native PAGE (figures not shown) unlike that of peanut [22]. RNA synthetic assays of the whole-gel eluted isoenzymes gave a series of predominantly low molecular weight

Table 1. Field weather conditions from transplanting to harvesting of the *Phyla dulcis* leaves[†].

Month	Rainfall (mm)	Maximum daily temperature (˚C)	Minimum daily temperature (˚C)	Relative humidity (%)	Solar radiation (lang)	Wind speed (mph)
March	0.61 ± 2.38[‡]	21.3 ± 4.6	7.4 ± 5.7	79.2 ± 14.9	371.6 ± 102.0	7.7 ± 2.7
April	3.5 ± 6.9	24.0 ± 4.2	12.2 ± 5.3	89.5 ± 9.1	353.8 ± 146.6	7.8 ± 2.9

[†]The weather data were obtained from the USDA-NRCS Scan Station on the Prairie View A & M University farm a few meters from the *Phyla* plot; [‡]Monthly mean and standard deviation.

(<500 nucleotides long) RNAs on agarose gels (figures not shown) irrespective of the pI values of the isoenzymes. Therefore, the acidic isoenzymes (Rotofor fractions 4 to 6) were combined, neutral isoenzymes (Rotofor fractions 7 and 8) were combined, and basic isoenzymes (Rotofor fractions 9 to 12) were combined and the three composite samples were applied for the double differential display analyses.

3.3. Differential Display of GDH-Synthesized RNAs of *P. dulcis*

The purpose of the differential display RF-PCR of the GDH-synthesized RNAs was to permit easy molecular biology amplification, isolation, and bioinformatics characterization of the cDNA fragments homologous to *P. dulcis* mRNAs as were done previously [21]. The double differential display patterns of the cDNAs (**Figures 2-4**) showed that the RNAs synthesized by the acidic, neutral, and basic isoenzymes of *Phyla* GDH were structurally different isomers in agreement with the binomial subunit compositions in the hexameric isoenzymes [21]-[25]. All the 64 display PROBES of Display Systems Biotech, Vista, CA, USA were studied. Based on the subsets of cDNA fragments obtained, the PCR priming by display PROBES 1, 4, and 18 (**Figures 2-4**) showed that the neutral isoenzymes of GDH were more active in RNA synthesis than the acidic and basic isoenzymes. The display patterns obtained in replicate RF-PCR experiments showed complete identity per display PROBE. Similarly, the differential band patterns for GDH-synthesized RNAs were different from those for mRNAs [26]. Therefore the synthesis of RNA by *P. dulcis* GDH was specific and reproducible reactions. cDNAs of GDH-synthesized RNAs are important Northern hybridization tools for monitoring and quantitation of the abundances of mRNAs that are homologous to them [12]-[17].

3.4. Messenger RNA Targets of *P. dulcis* GDH-Synthesized RNAs

To assign functions to the cDNA fragments, their sequences were used to search the GenBank databases with the BLASTN etc. algorithms. An overview of the mRNA targets homologous to the differential cDNA fragments (**Figures 2-4**) showed that overwhelmingly the mRNAs encoding the enzymes of primary metabolism were the targets of the RNAs synthesized by the acidic GDH isoenzymes; whereas predominantly the mRNAs encoding the enzymes of natural products metabolism were the targets of the RNAs synthesized by the neutral and basic GDH isoenzymes. These are important molecular differences and similarities that would allow environmental conditions to be focused either on the primary or terpenoid metabolism of the plant species.

Differential cDNA band 1.02 (**Figure 2**) yielded several isomeric cDNAs (**Table 2**). They showed sequence homologies with mRNAs encoding *Phyla* primary metabolic enzymes including dehydroquinate dehydratase, small subunit of ribulose-1,5-bisphophatase carboxylase, cytochrome P-450 reductase, granule-bound starch synthase, pyruvate kinase, glucose-6-phosphate dehydrogenase, protoporphyrinogen oxidase, phosphoenol pyruvate carboxylase, quinolinate phosphirobosyltransferase, photosystem II protein, and coumarate: coenzyme A ligase. The mechanism of GDH-synthesized RNA is that they silence mRNAs homologous to them [16] [24]. All the mRNAs homologous to cDNA fragments 1.02 (**Figure 2**) have different molecular weights therefore making them very easy to identify on Northern blot analyses. This is a considerable advantage in target mRNA profiling and modeling of biochemical pathways [14] [17]. Differential cDNA fragment 1.03 (**Figure 2**) was homologous to mRNA encoding peroxidase. The metabolic probes embodied by the cDNA fragments 1.02 and

Figure 1. *Phyla dulcis* growing in field plot in Texas summer conditions. There are green leaves, and older leaves that are purple.

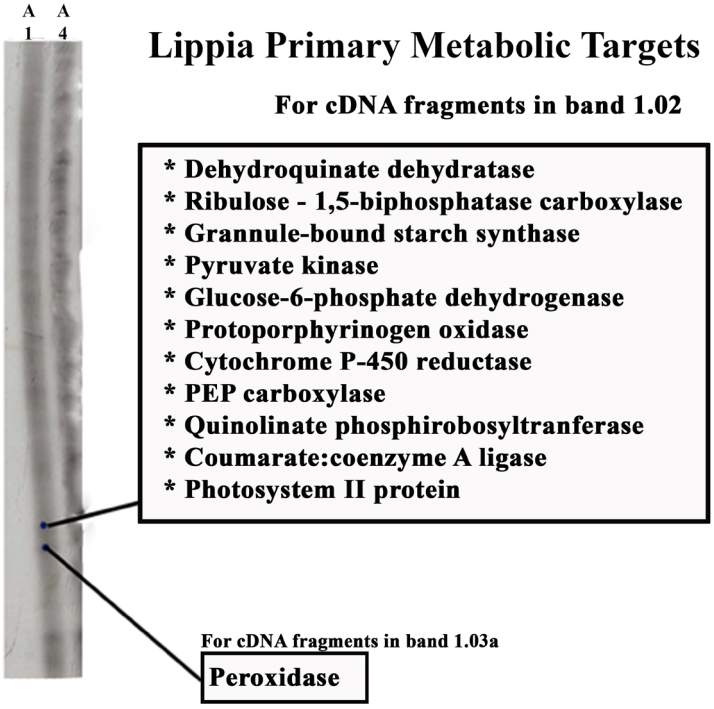

Figure 2. Differential display of RNA synthesized by the GDH of *P. dulcis*. The RNA synthesized by the acidic isoenzymes (whole gel purified Rotofor fractions 4-6 were combined = A) was used. After cDNA preparation, the restriction fractions were amplified by Display System's Double Differential-PCR method and fractionated on sequencing PAGE. Lanes A1, A4 were for display PROBEs 1 and 4 respectively as PCR primers. The indicated fragments were sequenced.

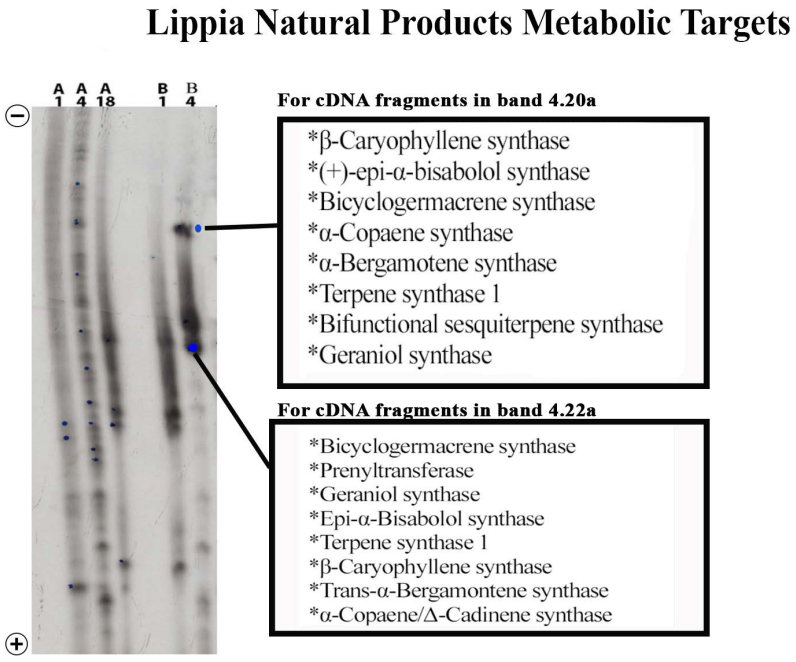

Figure 3. Differential display of RNA synthesized by the GDH of *P. dulcis*. The RNA synthesized by the acidic isoenzymes (whole gel purified Rotofor fractions 4-6 were combined = A), and neutral isoenzymes (whole gel purified Rotofor fractions 7 & 8 were combined = B) were used. After cDNA preparation, the restriction fractions were amplified by display System's Double Differential-PCR method and fractionated on sequencing PAGE. Lanes A1, A4, A18 were for display PROBEs 1, 4 and 18 respectively as PCR primers for the acid isoenzyme synthesized RNA; lanes B1, and B4 were for display PROBES 1 and 4 respectively for the neutral isoenzyme synthesized RNA. The indicated fragments were sequenced.

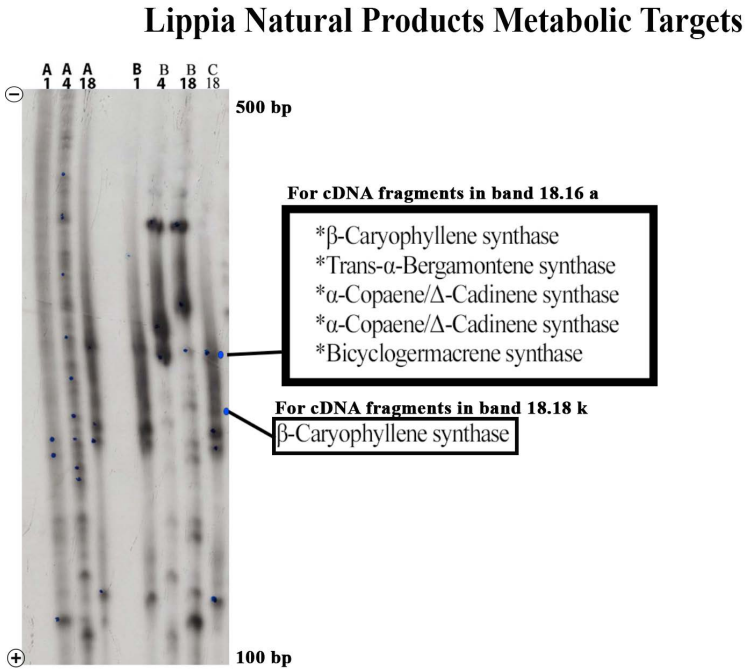

Figure 4. Differential display of RNA synthesized by the GDH of *P. dulcis*. The RNA synthesized by the acidic isoenzymes (whole gel purified Rotofor fractions 4-6 were combined = A), neutral isoenzymes (whole gel purified Rotofor fractions 7&8 were combined = B), basic isoenzymes (whole gel purified Rotofor fractions 9-12 were combined = C) were used. After cDNA preparation, the restriction fractions were amplified by display System's Double Differential-PCR method and fractionated on sequencing PAGE. Lanes A1, A4, A18 were for display PROBEs 1, 4 and 18 respectively as PCR primers for the acid isoenzyme synthesized RNA; lanes B1, B4, and B18 were for display PROBES 1, 4, and 18 respectively for the neutral isoenzyme synthesized RNA; lane C18 was for display PROBE 18 for the basic isoenzyme synthesized RNA. The indicated fragments were sequenced.

1.03 constitute important molecular tools for monitoring and regulating *P. dulcis* mRNA targets in photosynthesis (**Figure 1**). The dual importance of GDH-synthesized RNAs is that *in vivo* they silence mRNAs homologous to them; and *in vitro* they serve as reliable probes in Northern hybridization analyses [12]-[16].

Complementary DNA fragments 4.20 (**Figure 3**) showed sequence homologies with mRNAs encoding *Phyla* terpenoid metabolic enzymes including *β*-caryophyllene synthase, (+)-epi-*α*-bisabolol synthase, bicyclogermacrene synthase, *α*-copaene/*δ*-cadinene synthases, trans-*α*-bergamotene synthase, terpene synthase 1, bifunctional sesquiterpene synthase, and geraniol synthase (**Table 2**). These are some of the terpene synthases that have been characterized before [7] [11]; and the encoding mRNAs are related to the enzymes that synthesize some of the undesirable secondary metabolites of *Phyla* [4] [10]. Complementary DNA fragments 4.22 (**Figure 3**) similar to fragments 4.20 showed sequence homologies with mRNAs encoding *Phyla* terpenoid metabolic enzymes including in addition prenyltransferase (**Table 2**). All the mRNA homologous to cDNA fragments 4.20 and 4.22 possess different molecular weights therefore making them very easy to profile on Northern blot analyses. The metabolic probes embodied by cDNA fragments 4.20 and 4.22 constitute important molecular tools for monitoring and regulating *Phyla* biochemical targets in the study of the effects of agronomic management factors (fertilization, irrigation, pesticide tolerance) on secondary metabolism.

RNAs synthesized by the basic isoenzymes of *Phyla* GDH (**lane C-18** of **Figure 4**) gave differential cDNA fragmentation patterns similar to those of the RNAs synthesized by the neutral isoenzymes (**lanes B-1**, **B-4**, **B-18** of **Figure 4**). Complementary DNA fragments 18.16 (**Figure 4**) shared sequence homologies with mRNAs encoding *β*-caryophyllene synthase, *α*-copaene/*δ*-cadinene synthases, and trans-*α*-bergamotene synthase, bicyclogermacrene synthase (**Table 2**) all of which are molecular targets in natural products metabolism. Complementary DNA fragments 18.18 (**Figure 4**) shared sequence homology with mRNAs encoding *β*-caryophyllene synthase (**Table 2**) therefore it will complement fragments 18.16 in Northern profiling of *Phyla* total RNAs. RNA differential display (**Figures 2-4**) is a sensitive, reproducible, and versatile technology for profiling different RNAs simultaneously [26].

Table 2. Some cDNA sequences of the RNAs synthesized by the GDH of medicinal plant *Phyla dulcis*, their homologous mRNAs, and the encoded enzymes.

	Enzymes and mRNA accessions	cDNA sequences of the GDH-synthesized RNAs homologous to the mRNAs
a	3-dehydroquinate dehydratase/shikimate dehydrogenase isoform ID: gb|AY578144.1|	gggtgcatagcggcgcgaattcgcccttactggtctcgtagactgcgtacccgataatcctcagcaaat gacaacgtggctggccgggcaagcctcggaccccgtaaagcgcgcgtccttgttgacgcacttttccc tcaaggatcaagctgacagaacgttttcagacggcgtacgtcagaccctgcaggggatgggaaactg gtccgatgcgcaggcatcaggcaatgctttccttgcgagcctcaacggttgggtgccggatcgtttcatc gctgttgagcagttgcacggtgatccttcggtcaggactcata
b	chloroplast ribulose 1,5-bisphosphate carbox-	tgagcgtattgcggncgcgnnatcgcccttgatgagtcctgaccgatataacgcgaagaaccttacca
c	ylase/oxygenase small subunit ID: gb|KM025319.1| cytochrome P-450 reductase ID: emb|X96784.1|	ggccggtacgcagtctacgagaccagtagcgatgagtcctgaccgggtacgcagtctacgagaccag tagtacgcagtctacgagaccagtagtacgcagtctacgagaccagttgtatgctgtttactggatgctttt gggcctggtagactgttccgtttagtgtaggtttgttgtgaacttggtgtggtgag
d	grannule-bound starch synthase ID: gb|EF584735.1|	ccttactggtctcgtagactgcgtacccggtcaggactcatcagtcaggactactggtctcgtagactgc gtacccggtcaggactcatcgctatatcggtcaggactcatcaa
e	pyruvate kinase (plastid isozyme) ID: emb|Z28374.1|	tatttttcttattcggcgccatactctctctctgtgctcacagaccgcgcaatactggtcgcgactcactact aatgggcccggaaagggtcttcgcgatatatcagtcacgactacatcaccttatagaccacgactcatc acatcagtcatgactcatcatatcagtaatgatcataatattggtcacgactcatcaagggagaattctatt aaacctgcaggactaccctctttaatgatggttaattatgatcttgtagta
f	cytosolic glucose-6-phosphate dehydrogenase ID: emb|AJ001769.1|	caggtgatagcggncgcgnattcgcccttgatgagtcctgaccgatatagcgatgagtcctgaccgat atagtgatgagtcctgaccgatatgcttttctccaggcgtaaaaaagcccgctcaattggcgggctgcta ttcttcggtcgggtacgcagtctacgagaccagta
g	protoporphyrinogen oxidase ID: gb|AF044128.1|AF044128	gggaatagcggatgcgcatgcgcactctctggtgtcgtagactgcctacccggttaggactcatccttta ctggtcgtgaagactgcgaactctgtgaggactcatcatcactggtctcgtagactgcgtacccggtca ggactcatcggtactggtctcgtagacagcttaccggcctgggaaggttcttcgtgttatatcggtcatga ttcatcaagcgcgaattctcc
h	phosphoenolpyruvate carboxylase ID: sp|P27154.1	ggcgtattagcggcgcgaattcgcccttactggtctcgtagactgcgtaatcggtcaggactcatcacta ctggtctcgtagactgcgtaccactggtctcgtagactgcgtactactggtctcgtagactgcatatcggt caggactcatca
i	photosystem II protein N: ID: NP_054528.1	ccttactggtctcgtagactgcgtacccggtcaggactcatcagtcaggactactggtctcgtagactgc gtacccggtcaggactcatcgctatatcggtcaggactcatca
j	4-coumarate:coenzyme A ligase. ID: dbj|D43773.1|	gtgcaatacggncgcgaattcgcccttactggtctcgtagactgcgtacccgatcaggactcatcgctat atcggtcaggctcatcactatatcggtcaggactcatca
k	peroxidase ID: dbj|AB044154.1|	gggggaaaaaacgggcgcgaattcgcccttactggtctcgtagactgcgatatcggtcaggactcatc actactggtctcgtagactgcgtacccggtcaggactcatcgctactggtctcgtagactgcgtacccgg tcaggactcatcgctatatcggtcaggactcatcatatcggtcaggactcatcaagggcgaattcgtttaa acctgcaggactagtccctttagtgagggttaattctgagcttggcgtaatcatggtcatagctgtttcctgt gtgaaattg
l	beta-caryophyllene synthase ID: gb|JQ731634.1	
m	(+)-epi-alpha-bisabolol synthase ID: sp|J7LH11.1|	
n	Bicyclogermacrene synthase ID: gb|JQ731633.1	
o	alpha-copaene/delta-cadinene synthase ID: gb|JQ731632.1|	gaggtaatagcggcgcgaattcgcccttactggtctcgtagactgcgtacccgatgcagaaggcggga aaacatgaaatgagcgtcaagcaggccgtgaaggttgccgagcttttgaagtgcaacccgatggaggt tatctgcggggtgatgtttcaccaggacgtaatggagcgggatttctggacggacattttccagcagaca
p	trans-alpha-bergamotene synthase ID: gb|JQ731635.1|	gtcaccgaaaacgaccgccgccactacttcaagaaggtttaggcaggctttcggtcaggactcata
q	Bifunctional sesquiterpene synthase 1 ID: sp|J7LP58.1|	
r	geraniol synthase ID: gb|ADK62524.1	
s	prenyltransferase alpha-subunit ID: emb|CDI30233.1|	ctggtctcgtagactgcgtacccgatgcagaaggcgggaaaacatgaaatgagcgtcaagcaggccg tgaaggttgccgagcttttgaagtgcaacccgatggaggttatctgcggggtgatgtttcaccaggacgt aatggagcgggatttctggacggacattttccagcagacagtcaccgaaaacgaccgccgccactact tcaagaaggtttaggcaggctttcggtcaggactcataagggcgaattcgtttaaacctgcaggact

3.5. Structure of RNAs Synthesized by *Phyla* GDH

The GDH-synthesized RNAs that were homologous to mRNAs encoding *Phyla* primary metabolic enzymes displayed extensive sequence similarities. The cDNA fragment (**Table 2**) homologous to the mRNA encoding granule-bound starch synthase shared 8-fold plus/minus sequence matches with the cDNA fragment (**Table 2**) homologous to the mRNA encoding glucose-6-phosphate dehydrogenase; 5-fold plus/minus sequence matches

with the cDNA fragment (**Table 2**) homologous to the mRNA encoding protoporphyrinogen oxidase; but 4-fold plus/plus sequence matches with the cDNA fragment (**Table 2**) homologous to the mRNA encoding 4-coumarate: coenzyme A ligase. The RNAs synthesized by GDH are isomeric in structure similar to the binomial subunit structure of the enzyme's hexamers [25]. Structural similarities between a GDH-synthesized RNA and several mRNAs in different biochemical pathways constitute the channels of cross-talks that differentially regulate or synchronize metabolism [12]-[16] through coordinate silencing as distinct from coordinate expression of transcripts. GDH is present in the cytoplasm, chloroplast, and mitochondria [27] thereby enabling the RNA it synthesizes to regulate different metabolic pathways.

There were also structural similarities between the RNAs synthesized by the acidic, neutral, and basic isoenzymes of *P. dulcis* GDH. The cDNA fragment (**Figure 2**) homologous to the mRNA encoding dihydroquinate dehydratase (**Table 2**) shared 3-fold matches two of which were plus/minus and the remaining one was plus/plus sequence matches with the cDNA fragment (**Table 2**) homologous to the mRNA encoding *β*-cryophyllene synthase, a natural products metabolic enzyme. The cDNA fragment (**Table 2**) homologous to the mRNA encoding granule-bound starch synthase (primary metabolism) shared 5-fold plus/plus sequence matches with the cDNA fragment (**Table 2**) homologous to the mRNA encoding bifunctional sesquiterpene synthase, a natural products metabolic enzyme. The geographic positioning of the cDNA fragments on the sequencing gel (**Figures 2-4**) whereby those resulting from the cathodal GDH isoenzymes (north side of the gel) regulated those from the anodal isoenzymes (south side) and *vice versa*; and those from the acidic GDH isoenzymes (west side) regulated those from the neutral and basic isoenzymes (east side) and *vice versa* describe the fidelity of the GDH-synthesized RNAs in the silencing of mRNAs *in vivo*, and the fool-proof characteristics of the cDNA fragments as Northern probes. The plus and minus strands of a cDNA fragment as two-probes-in-one potentially assure that the target mRNA will not be missed during Northern hybridization reaction. Furthermore, the multiple internal sequence repeats in the GDH-synthesized RNA assure tenacious crisscross binding of the cDNA fragment to the homologous mRNA thereby maximizing specificity and stringency through liquefaction reaction during Northern hybridization. Single-stranded nucleic acid probes lack the crisscross tenacious binding specificity and stringency embodied by GDH-synthesized RNAs. Geographic positioning on the sequencing gel landscape of the RNAs synthesized by GDH (**Figures 2-4**) would permit the biochemical selection of specific metabolic chemotypes of *P. dulcis* as was in the case of peanut [12]-[17]. Therefore, photosynthetic processes regulate secondary metabolism, suggesting that environmental conditions would be able to differentiate the metabolism of terpenoids to produce several *Phyla* chemotypes. Aside from the general fact that the initial substrates for secondary metabolism are sourced from primary metabolism, the cross-connections at the mRNA level (coordinated silencing) between primary and secondary metabolic pathways could potentially provide a practical approach for molecular regulation of terpenoid accumulation. The superimposition of a rainbow of environmental conditions on *Phyla* growth and development may manipulate the inter-mRNA cross-talks to the extent that special metabolic chemotypes arise some of which could be high hernundulcin thus illuminating possible alternative chemical treatment approaches [5] [6] for the cultivation of the medicinal plants.

By knocking down biomass and fatty acid metabolic pathways, GDH-synthesized RNAs enhanced the production of arachin-free low linoleic acid [14], and ultra-high resveratrol [17] peanut kernels. Similar technologies could be readily patterned for knocking down aspects of *Phyla* photosynthetic or monoterpene metabolism in order to enhance sesquiterpene accumulation. Several poor or disaster-afflicted rural communities exist worldwide [28]. Governments and humanitarian agencies are reaching out to alleviate the sociological and agricultural stresses of those communities. *Phyla dulcis* now grows luxuriantly in USA (**Figure 1**). *Phyla* species are money-makers [2] for small-scale farmers promoting rural economy in South Africa, India, China, and South America. High hernundulcin metabolic variants of the medicinal plants could enable limited resource farmers to increase their farm income thereby promoting rural economy world-wide besides complementing the industrial production of hernundulcin [1] [8] [9]. One of the major predispositions to obesity is the consumption of high calorie food. Sugar is one of the high calorie ingredients added in to food. The zero-calorie hernundulcins could possess the potential to minimize sugar intake while offering sweet taste to sweet-consumers. High-hernundulcin *Phyla*-based natural sweetener, similar to stevia could be popular in the health-conscious market place.

4. Conclusion

Phyla dulcis contains hernundulcin sesquiterpene zero-caloric sweetener and also bitter constituents including

camphor, limonene, terpineol, α-pinene, α-copaene, trans-caryophyllene, δ-cadinene, and α-bisabolol. There is yet no simple method to remove the undesirable components. Therefore, R & D are being focused to plant-based zero-caloric sweeteners. The aim was to characterize the mRNA targets that regulate the primary and terpenoid metabolic enzymes. Restriction fragment differential display polymerase chain reaction methodology showed that the RNAs synthesized by the glutamate dehydrogenase (GDH) of *Phyla dulcis* shared extensive sequence homologies with many mRNAs encoding dehydroquinate dehydratase, ribulose-1,5-bisphophatase carboxylase, granule-bound starch synthase, pyruvate kinase, glucose-6-phosphare dehydrogenase, protoporphyrinogen oxidase, cytochrome P-450 reductase, phosphoenol pyruvate carboxylase, quinolinate phosphirobosyltransferase, photosystem II protein, and coumarate:coenzyme A ligase in primary metabolism; and also β-caryophyllene synthase, (+)-epi-α-bisabolol synthase, bicyclogermacrene synthase, α-copaene/δ-cadinene synthases, trans-α-bergamotene synthase, terpene synthase 1, bifunctional sesquiterpene synthase, and geraniol synthase in terpenoid metabolism. GDH-synthesized RNAs are important because *in vivo* they coordinately silence appropriate mRNAs homologous to them, and their cDNAs are applied *in vitro* as high specificity maximum stringency probes to profile mRNA responses to environmental changes affecting primary metabolism and the accumulation of secondary metabolites. The extensive sequence similarities between GDH-synthesized RNA and several mRNAs encoding photosynthetic enzymes and terpene synthases suggested that secondary metabolism of *Phyla dulcis* could be regulated by photosynthesis at the mRNA level, an emerging subject that has not been widely exploited in plant biotechnology.

5. Ethical Compliance

The authors declare no conflict of financial interests. The research project was approved by the Institutional Review Board of the University; Prairie View A & M University, Texas A & M University, and USDA-NIFA Plan of Work; and supported by NIFA-USDA Allen Fund.

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