

Structural Properties of the RNA Synthesized by Glutamate Dehydrogenase for the Degradation of Total RNA

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Abstract

Glutamate dehydrogenase (GDH)-synthesized RNA, a nongenetic code-based RNA is suitable for unraveling the structural constraints imposed on the regulation (transcription, translation, siRNA etc.) of metabolism by genetic code. GDH-synthesized RNAs have been induced in whole plants to knock out target mRNA populations thereby producing plant phenotypes that are allergen-free; enriched in fatty acids, essential amino acids, shikimic acid, resveratrol etc. Methods applied hereunder for investigating the structural properties of GDH-synthesized RNA included purification of GDH isoenzymes, synthesis of RNA by the isoenzymes, reverse transcription of the RNA to cDNA, sequencing of the cDNA, computation of the G+C-contents, profiling the stability through PCR amplification compared with genetic code-based DNA; and biochemical characterization of the RNAs synthesized by individual hexameric isoenzymes of GDH. Single product bands resulted from the PCR amplification of the cDNAs of GDH-synthesized RNA, whereas several bands resulted from the amplification of genetic code-based DNA. The cDNAs have wide G+C-contents (35% to 59%), whereas genetic code-based DNA has narrower G+C-contents (50% to 60%). The GDH β homo-hexameric isoenzyme synthesized the A+U-rich RNAs, whereas the α 6, and α 6 homo-hexameric isoenzymes synthesized the G+C-rich RNAs. Therefore, the RNA synthesized by GDH is different from genetic code-based RNAs. *In vitro* chemical reactions revealed that GDH-synthesized RNA degraded total RNA to lower molecular weight products. Therefore, GDH-synthesized RNA is RNA enzyme. Dismantling of the structural constraints imposed on RNA by genetic code liberated RNA to become an enzyme with specificity to degrade unwanted transcripts. The RNA enzyme activity of GDH-synthesized RNA is ubiquitous in cells; it is readily induced by treatment of plants with mineral nutrients etc. and may simplify experimental approaches in plant

enzymology and molecular biology research projects.

Keywords

RNA Enzyme Synthesis, Nongenetic Code-Based RNA, Cloning Vector DNA, Touch-Down PCR, Frame Shift Sequence Homology

1. Introduction

Glutamate dehydrogenase (GDH EC 1.4.1.2) is a multi-subunit enzyme that polymerizes ribo-nucleoside triphosphates to produce RNA independent of any template [1] but dependent on the enzyme's binomial hexameric subunit composition, a property that is controlled by metabolic environments [2] [3]. Therefore, RNA synthesized by GDH is not genetic. Plant GDH synthesizes large quantities of RNA [4]. The same sequence and population of RNA are synthesized irrespective of the reducing or oxidizing reaction conditions. GDH is functional in all organisms. The metabolic nature of GDH-synthesized RNA provides the opportunity to study the chemical contrasts between genetic code-based and nongenetic code-based RNAs for the differentiation of their biological functions. The GDH-synthesized RNA shares sequence homologies with many mRNAs [1]. They regulate total RNA abundance [5]. GDH-synthesized RNAs have been reverse-transcribed to cDNA and sequenced, leading to large scale preparation of the silencing oligonucleotides, which are routinely applied as tools for monitoring mRNA concentrations, and metabolic networks that coordinate the biochemical pathways [4]. GDH isoenzymes have been induced in whole plants to synthesize selected sets of RNA that knocked out target mRNA populations thereby producing plant metabolic variants that are specialized in the accumulation of many metabolites of dietary importance. Specifically, allergen-free low linoleic acid peanuts [6], fatty acid-enriched peanuts [3], essential amino acid-enriched peanuts [7], ultra-high nutraceutical resveratrol contents of peanut seeds [8], shikimic acid-enriched *Phyla dulcis* plants [9]; and doubling of biomass yield [4] are some of the biotechnological milestones achieved by unleashing the mRNA degradation activity of GDH-synthesized RNA. The mechanisms of RNA enzyme, and transcript silencing are known [10] [11] [12], but the chemistry by which GDH-synthesized RNA (nongenetic code-based RNA) degrades total RNA (genetic code-based RNA) has not been discussed [5]. RNA-RNA interactions have been studied in detail [13] [14] [15], but the homologous alignment interactions in solutions, between nongenetic code RNA and genetic code-based RNA have not been studied. This paper opens a conversation on the *in vitro* reactions between GDH-synthesized RNA and total RNA moieties.

Following the biotechnological applications of GDH-synthesized RNA as enzyme, there is need to characterize their chemical properties as compared with genetic code-based RNA. There are many contrasts in the chemistry of coding

RNA and non-coding RNA [16] [17] [18] [19] [20]. Both coding and non-coding RNAs are however genetic. It is important that genetic code-based and nongenetic code-based RNAs are compared so as to begin to unravel the constraint and freedom imposed on metabolic regulation (siRNA silencing, transcription, replication, translation, genome structure etc.) by genetic code. Methods for investigating RNA generalized molecular properties revolve around the computation for G+C- and A+T-compositions and nearest neighbor base stacking interactions after RNA has been converted to cDNA [21] including algorithmic analyses for probable intramolecular binding sites [13]. Although G+C contents have been applied repeatedly in the description of nucleic acid structure [16] [17] [18] [20], the biochemical basis of the differential distribution of G+C in genomes has not been discussed. Other structural properties of nucleic acids are controlled by hydrogen bonding-related stabilization, melting and annealing temperatures, paramagnetic and electrostatic instability, interactions with protein enzymes and ligands [22] [23] [24]. Most of the properties including the G+C contents could be deduced by subjecting the cDNA to polymerase chain reaction (PCR) amplification, one of the experimental approaches presented hereunder. The structural differences between GDH-synthesized RNA and genetic code-based RNA illuminate the catalytic properties of GDH-synthesized RNA.

2. Materials and Methods

2.1. Treatment of Experimental Organism

Peanut (*Arachis hypogaea* Floor Runner cv.) seeds were sterilized in 5% alcohol solution for 10 min, rinsed with deionized water, and planted on moistened filter paper in five replicate petri dishes. The compositions of the mineral nutrients were: **N+N+N+K+S+S** (1 L of 75 mM NH₄Cl, 4 mM KCl, 100 mM Na₂SO₄); **N+P+K+K+K** (1 L of 25 mM NH₄Cl, 20 mM Na₃PO₄, 12 mM KCl); **N+P+S+S+S+S** (1 L of 25 mM NH₄Cl, 20 mM Na₃PO₄, 200 mM Na₂SO₄); **N+P+P+P** (1 L of 25 mM NH₄Cl, 60 mM Na₃PO₄). The control was moistened with distilled water. About 8 - 10 seeds were planted per petri dish and allowed to germinate in the greenhouse temperature of 24°C - 28°C, and relative humidity of 70% - 80% under Texas month of May temperature and sunlight conditions. The greenhouse was shaded 50% with a shade cloth. Filter papers were changed and re-wetted with fresh mineral solutions daily. The applied mineral salt compositions were based on stoichiometric combinations to mimic the binomial subunit polypeptide compositions of GDH isoenzymes [25] [26] and to interact with seeds' target molecules in molar ratios. After emergence of full plumule, germination was stopped; and the seedlings were stored in -30°C freezer.

2.2. Total RNA

Total RNA was extracted from peanut seedlings using the acidic phenol/chloroform

(pH 4.5) method [27].

2.3. Purification of GDH Isoenzymes

GDH was purified from the **N+N+N+K+S+S-treated** peanut seedlings (20 g) by homogenization at 4°C with 100 mL of buffer [28] containing 5 units per mL of each of RNase A, and DNase 1. The homogenate was centrifuged (5000 ×g, 15 min, 4°C), and the supernatant was frozen at -80°C, thawed at room temperature in order to fracture the mitochondria and to assure that DNA and total RNA have been degraded. After re-centrifugation (9000 ×g, 4°C, 30 min), the supernatant was subjected to fractional ammonium sulfate precipitation, preparative-scale isoelectric focusing (IEF; Rotofor, Bio-Rad, Hercules, USA) followed by dialysis of the fractions as described before [4]. Rotofor fractions (0.2 mL) were purified by native 7.5 % polyacrylamide gel electrophoresis (PAGE) (100 V, 16 h, 4°C) to remove other proteins, and nucleic acid contaminations. GDH isoenzymes were eluted (30 min, 100 V) from the electrophoresed gel with 0.05 M solution of Tris base, at subzero temperature in the elution chamber, of mini-whole gel eluter (Bio-Rad) as described before [29]. The eluted GDH charge isomer fractions were collected separately per eluter channel, and stored at -30°C.

2.4. RNA Synthesis by GDH

RNA was synthesized in the amination direction in cocktails containing solution of 0.87 mM NH₄Cl, 3.5 mM CaCl₂, 10.0 mM α -ketoglutarate (α -KG), 0.23 mM NADH, 0.6 mM each riboNTP, 5 U of RNase inhibitor, 5 U of DNase 1, and 5.0 μ g actinomycin D. The reaction was started by adding 0.2 mL of cryo-electrophoretically purified GDH charge isomers containing about 500 μ g protein per mL as described before [29]. The final volume of the reaction was brought to 0.4 mL and pH 8 with 0.1 M Tris-HCl solution. Reactions were incubated at 16°C overnight (16 h) and stopped by phenol-chloroform (pH 4.5) extraction of the GDH. The RNA was precipitated with ethanol. RNA synthesis was carried out three times to verify the reproducibility of the results.

2.5. Restriction Fragment Differential Display PCR

cDNAs were synthesized with 2 μ g of each product RNA synthesized by the GDH charge isomers purified from the **N+N+N+K+S+S-treated** seedlings, using random hexamer primers. The product cDNA (5 μ g) was digested with Taq 1 restriction enzyme (5 Units) for 2 h at 65°C. In order to compare with the chemistry of genetic code-based DNA, pCR4-TOPO vector DNA (5 μ g) was similarly digested in another micro tube with Taq 1 restriction enzyme (5 Units). Adaptors, ³²P-labeled extension primers, and the selective display PROBE combination (Display Systems Biotech, Vista, CA USA) were ligated to the ends of the restriction fragments. The nucleotide sequences of the adaptors, extension

primers, and display PROBES are among the proprietary information of their manufacturer, Display Systems Biotech, Vista, CA USA.

The template DNA (0.5 µg) was amplified according to the 'touch-down' restriction fragment double display (RF-DD) PCR methods of Display Systems Biotech, Vista, CA, USA. Initial denaturation was 96°C for 1 min. For the first 10 cycles: denaturation was at 96°C for 30 sec., annealing was at 60°C for 30 sec, for the first cycle, then reduced the annealing temperature 0.5°C each cycle until an annealing temperature of 55°C was reached after 10 cycles; extension was at 72°C for 1 min. PCR was continued another 25 cycles: denaturation (96°C, 30 sec), annealing (55°C, 30 sec), extension (72°C, 1 min); final elongation (72°C, 5 min). All the 64 display PROBES in the Display Systems Biotech kit were used in the differential display PCR. The differential bands/products were visualized by autoradiography following polyacrylamide sequencing gel electrophoresis. Selected cDNA, and vector DNA fragments were sub-cloned into pCR4-TOPO vector and transformed into TOP10 One Shot Chemically Competent (non-pathogenic) *Escherichia coli* (Invitrogen, Carlsbad, CA), followed by overnight growth on kanamycin selective plates. Up to 15 positive transformant colonies were picked per plate and cultured overnight in LB medium containing 50 µg/mL of kanamycin. Plasmids were purified with a plasmid kit (Novagen, Madison, WI), and the insert cDNA or vector DNA fragment of selected recombinant plasmids were sequenced with T7, and T3 primers by MWG Biotech Inc., High Point, North Carolina, USA. The sequenced plasmids were amplified by 'touch-down' PCR using M13 primers (Invitrogen Life Technologies, California); separated by agarose gel electrophoresis; the product bands were UV-visualized, and photo-documented.

2.6. Reaction between GDH-Synthesized RNA and Total RNA

GDH-synthesized RNA and total RNA preparations were normalized to a concentration of 5 µg/µL in 0.1 M Tris-HCl buffer solution pH 8.0. The reactions were conducted in 8 numbered micro-centrifuge tubes placed on ice. Tube 1 contained 100 µg of total RNA of control peanut, and 100 µg of the GDH-synthesized RNA of N+N+N+K+S+S-treated peanut. Tube 2 contained 200 µg of total RNA of control peanut. Tube 3 contained 100 µg of total RNA of N+P+K+K+K-treated peanut and 100 µg of the GDH-synthesized RNA of N+N+N+K+S+S-treated peanut. Tube 4 contained 200 µg of the GDH synthesized RNA of N+N+N+K+S+S-treated peanut. Tube 5 contained 100 µg of the total RNA of N+P+S+S+S+S-treated peanut and 100 µg of the GDH synthesized RNA of N+N+N+K+S+S-treated peanut. Tube 6 contained 200 µg of the GDH synthesized RNA of N+N+N+K+S+S-treated peanut. Tube 7 contained 100 µg of the total RNA of N+P+P+P-treated peanut and 100 µg of the GDH synthesized RNA of N+N+N+K+S+S-treated peanut. Tube 8 contained 200 µg of the total RNA of control peanut. To each tube, 1 µL of 0.6 mM ribo-NTP mix was added; and the final volume of the reaction was brought to 50 µL with 0.1 M

Tris-HCl buffer solution pH 8. Tubes 1, 2, 3, 4, 5, and 7 were thermo-cycled: pre-heat (96°C, 30 sec). Then 40 cycles of cool (5°C, 1 min), warm (37°C, 2 min). Final storage (5°C). Tubes 6, and 8 were left on ice. The extent of reaction was demonstrated by 2% agarose gel electrophoresis of 7 µL of each reaction solution. The gel was stained with ethidium bromide solution; and photographed. RNA band intensities were digitalized using UN-SCAN-IT gel digitalizing software (Silk Scientific, Inc., Orem, Utah, USA).

2.7. Structural and Functional Characterization of RNAs

To assign putative functions to the RNAs synthesized by GDH (RNA enzyme), their cDNA sequences were used as queries to search the NCBI nucleotide-nucleotide (excluding ESTs) BLAST (blastn) for peanut taxid 3818 database (*Arachis hypogaea*) [30]. Similarly, to identify the fragments of the cloned pCR4-TOPO vector DNA, the sequenced fragments were used as queries to search the NCBI nucleotide-nucleotide blastn. Putative functions were assigned to alignments with the highest scores, and in consideration of the molecular weight of the corresponding agarose gel electrophoresis bands of the PCR products. To compare sequence homologies among the GDH-synthesized RNAs, the NCBI BLASTN 2 Sequences alignment algorithm was used [31].

2.8. DNA Physical Properties

DNA sequences were applied to the Kibbe equations (<http://www.basic.northwestern.edu/biotools/oligocalc.html>) to count the cytosines and guanines accurately and to calculate the G+C contents, nearest neighbor, and melting temperatures (T_m) taking into account base stacking energy.

3. Results and Discussion

3.1. G+C Contents and Amplification Uniformity

Single product bands resulted from the PCR amplification of the insert cDNA of GDH-polymerized RNA (**Figure 1**), whereas multiple product bands resulted from the amplification of vector insert DNA (genetic code-based DNA), despite that the T_m applied in the “touch-down” PCR was stringent (**Table 1**), and the PCR protocol was conducted at the same time, with same cocktail of reagents, under same conditions. Therefore, there was amplification stability and uniformity for the cDNAs of GDH-synthesized RNA and amplification instability for the genetic code-based DNA. “Touchdown” PCR is recommended for circumventing incomplete priming [32] [33] [34], and for increased specificity and sensitivity in PCR amplification [35], but many of the recombinant plasmids (**Figure 1** lanes 14, 22, 23, 24, 27, and 29) with vector DNA inserts did not produce a main PCR product band thus suggesting that the chemistry of the insert vector fragment interfered with the complete melting dissociation of the DNA strands. Such incomplete melting dissociation of strands could arise from very

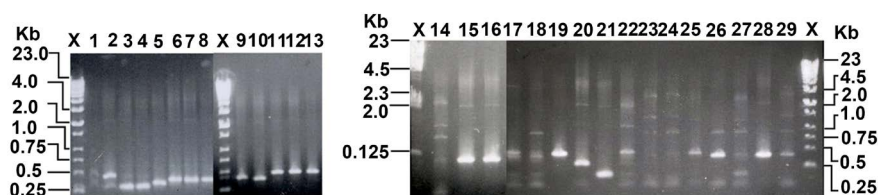


Figure 1. Polymerase chain reaction amplification of the cDNAs of GDH-synthesized RNA (nongenetic code-based) compared with that of genetic code-based DNA. The amplification was by touch-down PCR, after which equal aliquots were electrophoresed on 2% agarose gel containing ethidium bromide. The gel was photographed after electrophoresis. The cDNAs of GDH-synthesized RNA are lanes 1-13, 15-20. Genetic code-based DNAs are lanes 14, 21-29. X is DNA molecular weight marker.

Table 1. Some thermodynamic properties of the cDNA of GDH-synthesized RNA contrasted with those of genetic code-based (vector) DNA fragments.

Plasmid Inserts	Length (base pairs)	GC Content %	Nearest Neighbor °C	T _m basic °C	Comment
1	143	57.0	84.92	83.7	A
2	313	35.5	79.86	77.3	A
3	153	44.0	79.98	78.5	A
4	153	51.0	82.27	81.4	A
5	191	54.0	83.76	83.5	A
6	243	50.0	84.49	82.5	A
7	243	50.0	84.49	82.5	A
8	243	50.0	84.49	82.5	A
9	261	54.0	85.39	84.5	A
10	241	49.8	84.45	82.5	A
11	241	49.8	84.45	84.1	A
12	334	52.0	84.98	84.1	A
13	319	59.0	87.47	86.8	A
14	146	59.0	86.18	84.4	A
15	308	35.0	79.70	77.0	A
16	312	35.3	79.88	77.2	A
17	101	54	83.39	80.6	A
18	69	55	78.24	77.7	A
19	341	37	80.79	78.3	A
20	121	55	82.99	82.0	A
21	159	55	84.27	83.3	B
22	302	50	85.17	83.3	B
23	199	59.0	87.74	85.8	B
24	365	50.7	86.28	84.0	B
25	365	51.2	86.37	84.0	B
26	188	59.0	87.74	85.8	B
27	292	51.0	84.67	83.5	B
28	166	59.0	86.18	84.4	B
29	442	60.0	89.4	87.8	B

a = cDNA of GDH-synthesized RNA (nongenetic code-based). b = genetic code-based DNA.

high nearest neighbor stacking base interactions, and high G+C compositions. The cDNAs of GDH-synthesized RNA varied their G+C contents broadly covering a range from 35% to 59%, whereas the vector coding DNA fragments varied their G+C contents for a narrower range from 50% to 60% (**Table 1**). Therefore, RNA synthesized by GDH is a modified RNA, its cDNA exhibiting chemical properties different from genetic code-based DNA.

The high G+C contents of the vector DNA (**Table 1**) are in agreement with previous observations [20] [36] [37] [38] that the lengths of coding DNA segments in the genomes are under structural constraints associated with their higher G+C contents compared with the poorer G+C regions [39]. But high G+C contents of DNA increase stability of genes [37]. Previous research on the importance of G+C contents compared the G+C-rich with the G+C-poor regions [36] of genetic code-based DNA. The comparisons presented here between the G+C contents of coding DNA sequence and the cDNA of GDH-synthesized RNA are at the base line for further unraveling of nucleic acid chemistry and function. Therefore, coding function imposed higher G+C contents-related structural constraint on genome, whereas GDH synthesis of RNA minimized that G+C-related structural constraint.

The nearest neighbor interaction is a major factor that affects the stability of nucleic acid, the A.T pairing being always destabilizing [21]. The nearest neighbor effects increased the T_m values to the same extent for both the cDNAs of GDH-synthesized RNA and genetic code-based DNA inserts (**Table 1**). The wider range (35% - 59%) in the G+C contents of the cDNAs of GDH-synthesized RNA compared with the narrower range (50% - 60%) in the vector DNA inserts suggests that cDNAs of GDH-synthesized RNA possess higher A+T contents which would destabilize at least some local sections in the double helix of the cDNAs. Local helical conformational changes have been reported to exercise biological functions [40]. Such relative difference in DNA stability may explain the uniformity of PCR amplification products for the cDNAs of GDH-synthesized RNA in contrast to the nonuniformity of the PCR products of vector DNA inserts (**Figure 1**). DNA polymerase could therefore be more active in the PCR of the cDNAs of GDH-synthesized RNA than in the genetic code-based vector DNA fragments.

It has been suggested that the RNA synthesized by GDH might function in the regulation of mRNA abundance through homologous sequence-mediated RNA interference activity [4] [6] [7]. In describing the features which are correlated with silencing efficiency, Chan *et al.* [16] identified the importance of low G+C-contents of the siRNA, suggesting that siRNA G+C-content negatively correlated with RNAi efficiency. Liu *et al.* [19] calculated that efficient siRNAs tend to be A+U-rich (G+C content is 44.0% to 47.8%), whilst inefficient siRNAs tend to be G+C-rich (GC content is 52.7% to 55.2%). The lower G+C-contents of the cDNAs of GDH-synthesized RNA (**Table 1**) explain the efficiency of GDH-synthesized RNA as siRNAs. The homologous target sequences

in the mRNA for the binding of the GDH-synthesized RNA are also A+U-rich. Conversely, the higher G+C-contents of genetic code-based DNA (**Table 1**) could explain some of their inefficient siRNA activities [41]. The target sequences in the mRNA for the binding of the genetic code-based siRNA are also G+C-rich. Some publications have stated that the high G+C-contents of genetic code-based siRNA constructs may appear to be the stumbling block in the medical or pharmaceutical application of efficient siRNA technology compared with antisense technology [19] [42]. GDH-synthesized RNA helps to circumvent some of genetic code-based siRNA structural constraints because GDH implements the synthesis in response to the prevalent metabolic environment of the plant, tissue, and cells. We have modified the metabolic environment of several crop species and succeeded in knocking out specific toxic and anti-nutritional components [3] [4] [6] [9]. Also, mammalian GDH synthesizes RNA.

The differences between the G+C-contents of genetic code-based DNA and nongenetic code-based DNA (**Table 1**), and in the amplification uniformity of nongenetic code-based DNA (**Figure 1**) imply that genetic code-based DNA possess paramagnetic and electrostatic properties [22] [23] [24] that are different from those of nongenetic code-based DNA. Therefore, when GDH-synthesized RNA (the RNA enzyme) aligns in the correct orientation and interacts with its homologous mRNA (genetic code-based RNA) during silencing biochemical reaction, the force of the collusion between the different paramagnetic-dielectric RNA molecules could be so considerable that the resultant energy could liquefy total RNA, the lesser stable of the two types of RNA (**Figure 2**).

3.2. Reaction between Genetic Code-Based RNA and Nongenetic Code-Based RNA

The reactions between total RNA and the RNA synthesized by GDH resulted to total RNA degradation (**Figure 2**). Digital pixelated comparison of the extents of reactions in lanes 1 (**Figure 2**: reaction between total RNA of control peanut and the RNA synthesized by the GDH of N+N+N+K+S+S-treated peanut), 2 (total RNA of control peanut, after thermal cycling), 4 (RNA synthesized by the GDH of N+N+N+K+S+S-treated peanut, after thermal cycling), 6 (RNA synthesized by the GDH of N+N+N+K+S+S-treated peanut, before thermal cycling), and 8 (total RNA of control peanut, before thermal cycling) showed that new molecular weight degraded bands lower than the 5S RNA band appeared in lane 1.

Comparison of lanes 3 (**Figure 2**: reaction between total RNA of N+P+K+K+K-treated peanut and RNA synthesized by the GDH of N+N+N+K+S+S-treated peanut), 2 (total RNA of control peanut, after thermal-cycling), 4 (RNA synthesized by GDH of N+N+N+K+S+S-treated peanut, after thermal cycling), 6 (RNA synthesized by GDH of N+N +N+K+S+S-treated peanut, before thermal cycling), and 8 (total RNA of untreated control peanut, before thermal cycling) also showed the emergence of new degraded RNA bands between the 16S and 5S rRNA bands that were absent from the GDH-synthesized RNA and total RNA in lane 3.

Interaction Between Total RNA and GDH-synthesized RNA

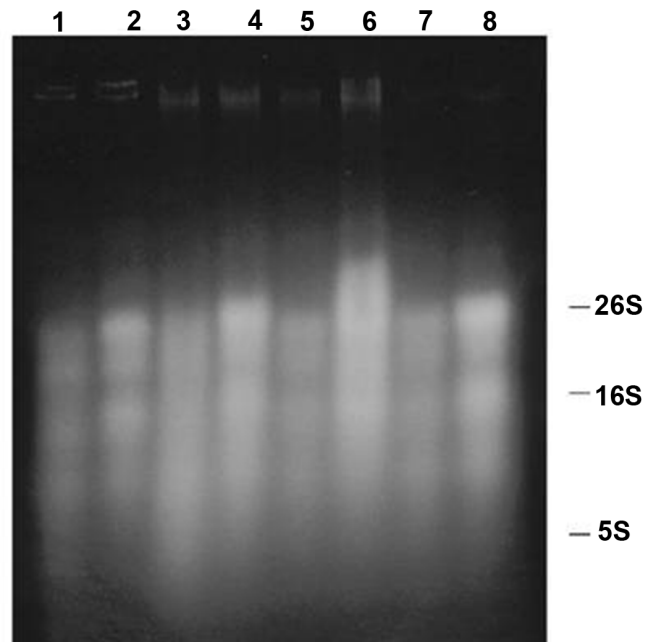


Figure 2. *In vitro* reaction between GDH-synthesized RNA and total RNA. The GDH-synthesized RNA enzyme activity was demonstrated in 8 test tubes. Reaction 1 (lane1) is total RNA of control peanut mixed with the GDH-synthesized RNA of N+N+N+K+S+S-treated peanut. Reaction 2 (lane 2) is control's total RNA. Reaction 3 (lane 3) is total RNA of N+P+K+K+K-treated peanut mixed with the GDH-synthesized RNA of N+N+N+K+S+S-treated peanut. Reaction 4 (lane 4) is the GDH-synthesized RNA of N+N+N+K+S+S-treated peanut. Reaction 5 (lane 5) is the total RNA of N+P+S+S+S+S-treated peanut mixed with the GDH-synthesized RNA of N+N+N+K+S+S-treated peanut. Reaction 6 (lane 6) is the GDH-synthesized RNA of N+N+N+K+S+S-treated peanut. Reaction 7 (lane 7) is the total RNA of N+P+P+P-treated peanut mixed with the GDH-synthesized RNA of N+N+N+K+S+S-treated peanut. Reaction 8 (lane 8) is the total RNA of control peanut. Reactions 1, 2, 3, 4, 5, and 7 were thermo-cycled; reactions 6 and 8 were not. After reaction, equal aliquots of reactions were electrophoresed (2% agarose gel), then the gel was photographed.

Similarly, reaction 5 (**Figure 2**: lane 5) between the total RNA of **N+P+S+S+S+S**-treated peanut and the RNA synthesized by the GDH of **N+N+N+K+S+S**-treated peanut demonstrated the considerable loss of RNA bands compared with the controls (lanes 2, 4, 6, and 8).

Again, reaction 7 (**Figure 2**: lane 7) between the total RNA of **N+P+P+P**-treated peanut and the RNA synthesized by the GDH of **N+N+N+K+S+S**-treated peanut displayed a vivid loss of RNA bands compared with the controls (lanes 2, 4, 6, and 8).

The reactions depicted in lanes 1, 3, 5, and 7 in the absence of protein enzymes suggested that the GDH-synthesized RNA acted as enzyme to degrade transcripts that shared sequence homologies with it because comparison of lanes

2 and 8 showed that the thermal cycling did not degrade total RNA, but lanes 4 and 6 showed that GDH-synthesized RNA was sensitive to thermal cycling, the high molecular weight bands above 26S rRNA in lane 6 being absent after thermal cycling (lane 4) which could have resulted from their thermal instability due to the high A+U content of GDH-synthesized RNA (Table 1). The thermal cycling temperatures applied in this study did not exceed 37°C in order to avoid RNA H-bond breakage during the RNA degradation reaction. The cooling of reaction temperature to 5°C was designed to allow the GDH-synthesized RNA to align with homologous sequences in total RNA moieties. Temperature was raised to 37°C to allow RNA degradation reaction to occur. This suggests there could be chemical interactions in the alignment of homologous RNA sequences. Meyer [14], DiChiacchio *et al.* [13] applied computational methods to uncover possible interactions between two RNA molecules. Reactions (Figure 2) were thermo-cycled between 5°C and 37°C to increase the rate of homologous sequence alignment, total RNA degradation rate, and to continuously disaggregate the reaction products from the surfaces of the RNA enzyme. We seeded the reactions (Figure 2) by addition of ribo-nucleoside triphosphates to the reaction mix in order to displace the position of equilibrium further to the right.

The results (Figure 2) were obtained by cross-over reaction between total RNA from a treated peanut and the RNA synthesized by the GDH from a differently treated peanut. When the total RNA and the GDH-synthesized RNA were from the same treatment of peanut, there were no reactions (results not shown). This suggested that the total RNA population in peanut is in steady state relationship with the mechanisms that regulate the synthesis of RNA by the prevalent GDH isoenzymes. This is the biochemical basis of the production of plant metabolic variants (phenotypes) at will, via induction of GDH synthesis of RNA [4] [7] [8] [9] [26].

Again, the molecular responses of the different total RNAs of peanut to the same GDH-synthesized RNA (lanes 1, 3, 5, and 7) were different, further suggesting that each mRNA profile of the total RNAs was for a specific metabolic phenotype/variant [4].

Therefore, the cross-over reactions (Figure 2) were the *in vitro* demonstration of the *in vivo* degradation of transcripts by the GDH-synthesized RNA. It could be, but without knowing it, that the biochemical mechanism underlying the experimentations involving the treatments of whole plants, seedlings, organs, tissues or cell cultures with solutions of nucleophiles, electrophiles, mineral fertilizers, N-carboxymethyl chitosan, biochemical regulators, and agricultural chemicals are the induction of GDH-synthesized RNA enzyme activity to degrade the set of homologous mRNA as in Figure 2. And, the molecular responses of plant metabolism to the environment is a huge discipline area of plant enzymology and molecular biology research that is important to scientific investigations in plant systems, crop production, forestry, natural and environmental resources management, and horticulture. RNA as enzyme has been demonstrat-

ed in the splicing process [10] [43] [44] but not in the regulation of mRNA abundance through degradation by nongenetic code-based RNA.

3.3. Biochemical Synthesis of G+C and A+U Contents of RNA

In vitro demonstration of the function of GDH-synthesized RNA as enzyme begets a conversation on the structure of the RNA (Table 2 and Table 3). The RNA oligonucleotides synthesized by each GDH hexameric isoenzyme illuminated the chemical frequency of G+C contents because instead of comparing the isoelectric point (pI) values of the polypeptides, GDH biological function is visualized in terms of nucleic acid sequence repeats and homologies (Table 2) similar to genetic code sequence repeats. GDH has a non-allelic gene structure consisting of three different subunit polypeptides [1]. The gene (GDH₁) encoding the more acidic subunits (a, and α) is heterozygous, and co-dominant; and the gene (GDH₂) encoding the less acidic subunit (β) is homozygous. The binomial distribution pattern of the 28 hexameric isoenzymes [45] is a protein population array that displays the subunit relationships on the native polyacrylamide gel landscape. The purification of active GDH isoenzymes from the slab of native acrylamide gel was made possible by the subzero electrophoretic fractionation processes that preserved the structural integrity and catalytic function of each hexameric isomer. Systematic pair-wise sequence homology comparisons [31] showed that the RNA (#19) synthesized by GDH β 6 homo-hexameric isoenzyme did not share sequence match with the RNA (#18) synthesized by GDH α 6 homo-hexameric isoenzyme, and did not share sequence match with the RNA (#17) synthesized by GDH α 6 homo-hexameric isoenzyme (Table 2 and Table 3). This is surprising because the three subunit polypeptides (a, α , and β) of the enzyme share common antigenic properties [26]. However, the RNA (#18) synthesized by GDH α 6 homo-hexamer shared three-fold homology matches with the RNA (#17) synthesized by GDH α 6 homo-hexameric isoenzyme. Global homology comparisons of the GDH-synthesized RNAs (Table 2) of N+N+N+K+S+S-treated peanut showed that most were synthesized by hexameric isoenzymes that consisted of mixed ratios of a-subunit and α -subunit polypeptides; and less frequency of RNAs synthesized by mixed isoenzymes that consisted of the three subunit polypeptides. The RNAs synthesized by nucleotide-treated peanuts were more complicated in their primary structure because almost all of the 28 hexameric isoenzymes of GDH were induced [1]. Therefore, the repeated plus/plus and plus/minus sequence codes (alignments) among the GDH-synthesized oligonucleotide RNAs (Table 2) appear to be reminiscent of the possible rudiments of nucleic acid biological codes. Global homology comparisons (Table 2) also revealed that all the RNAs with low G+C contents were synthesized by the GDH β 6 homo-hexameric isoenzyme. Conversely, all the RNAs with high G+C contents were synthesized by the GDH hexameric isoenzymes enriched in a-subunit and/or α -subunit polypeptides. Therefore, in the polymerization of ribo-nucleoside triphosphates to produce RNA enzyme, the

Table 2. Binomial statistical sequence homologies of some RNAs synthesized by hexameric ($\alpha 6$, $\alpha 6$, $\beta 6$ etc.) isoenzymes of Peanut glutamate dehydrogenase.

GDH RNA	19 ($\beta 6$)	18 ($\alpha 6$)	8	4	13	20	1	5	7	10	9	15	16	2
19 ($\beta 6$)	---	no match	no match	no match	no match	no match	+/- match	no match	no match	no match	no match	+/+ match	+/- match	+/+ match
17 ($\alpha 6$)	no match	3 +/+ matches	6 +/+ matches	5 +/+ matches	4 +/- matches	3 +/+ matches	6 +/+ matches	4 +/+ matches	4 +/+ matches	4 +/+ matches	4 +/+ matches	no match	no match	no match
18 ($\alpha 6$)	no match	---	4 +/+ matches	4 +/+ matches	3 +/- matches	3 +/+ matches	4 +/+ matches	3 +/+ matches	4 +/- matches	4 +/- matches	6 +/- matches	no match	no match	no match
15	+/+ match	no match	no match										+/- match	
16	+/- match	no match	no match											
4	no match	4 +/+ matches	4 +/+ matches											
5	no match	3 +/+ matches	3 +/+ matches											
2	+/+ match	no match	no match											
1	+/- match	4 +/+ matches	4 +/+ matches											
20	no match	3 +/+ matches	7 +/+ matches											
10	no match	4 +/- matches	no match											

$\beta 6$ homo-hexameric GDH isomer selected ribo-ATP and UTP preferentially as substrates; whereas the $\alpha 6$, $\alpha 6$ and combinations thereof selected ribo-GTP and ribo-CTP preferentially as substrates. These biochemical considerations give strength to the repeatedly published over whelming preponderance of G+C-rich regions as against the lesser frequency of the A+T-rich regions in the genome [20] [36] [37]. GDH hexameric isoenzymes are being applied routinely for the synthesis of nucleic acid hybridization probes for studying the biochemical pathways of the recalcitrant medicinal plants, *Phyla dulcis* [9] [46]. Studies which applied genetic code-based hybridization probes instead [47] did not knock out the mRNAs encoding the monoterpene synthases, whereas studies with hybridization probes based on GDH-synthesized RNA enzyme have knocked out the mRNAs encoding the monoterpene synthases (Osuji unpublished results). This illustrates the efficiency of GDH-synthesized RNA enzyme.

The stoichiometric mineral solutions applied (N+N+N+K+S+S; N+P+K+K+K; N+P+S+S+S+S; and N+P+P+P) in peanut germination were selected because they were among those that did not delay the emergence of seedling radicle compared with water treatment. There are 299 stoichiometric combinations of mineral macronutrients that mimic the hexameric subunit structure of GDH. We have planted peanut seeds in the month of May in the tissue culture chamber,

Table 3. Some cDNA inserts and genetic code-based (vector) DNA inserts.**Plasmid 1: cDNA insert***Arachis hypogaea* oxalate oxidase (OxOxl) mRNA, EU024476.1TGAGTCCTGACCGATAGCGCAATGCGTTGAGTACCTTCAACGCCAAGAACGTCAACTACCAGCGCACGCCGCACTTCAAGAACAAA
CCCCGCACGCGGCATAGCGATGAGTCTGACCGGTACGCAGTCTACGAGACCAGTA.**Plasmid 2: cDNA insert***Arachis hypogaea* profilin (Ara h5) mRNA AF059616.1GATTTTATTTAGGAGTATTGGGAACGAATTGGAATGTAATAATATTGATTCATAGAGATCCAGAAGAAAAAGAATAATCTTCTACTT
TGAGAATAATAAAAAAGAAAAGTGTCAATTGGAACATGAAAACGTGACCTGACTGAATACTGGTCTCGTAGACTGCGTACCCGGT
CAGGACTCATCGCTACGTTAGCGTCTCTGAGGCGCGCTATTCTACAATCTTAAAAACCCCTGTCAACCCTTTAAATGCTTTTAAAGAC
AATGATTTGCGCTTCTTTCTGATTTCTTCTGGGGAGAAGAAACCCGTGGGCTGACGTTGCTGCGGGGCGCACITTTACAAGCCTTTGC
CTTACAGTTCAACGCCCTATCGGTCAGGACTCATAAGGGC**Plasmid 3: cDNA insert***Arachis hypogaea* ubiquitin-conjugating enzyme (UBC) mRNA, AY769917.2GAGTCTGACCGATAGTGAATAGGTGCTTGTGTTTCATGAGGCCTCCTTGATACTCATGAACTACAGATATTTGACGTCAAAAAATAAT
TCAAATAAGTTGTCCGACAATGCTGATGAGTCTGACCGGTACGCAGTCTACGAGACCAGTAAG**Plasmid 4: cDNA insert***Arachis hypogaea* type 2 metallothionein (MT2d) mRNA, DQ665256.1GAGTCTGACCGATAGGAGATCAAGGCACCCCATGTCTTGAGGGTGGGACGGTTATTGCTCAGGATAATAAAGGGCGGTTTCAGTT
CAAAGTGCCTGAGCTTAGTATAGCGATGAGTCTGACTGGGTACGCAGTCTACGAGACCAGTAAG**Plasmid 5: cDNA insert***Arachis hypogaea* mRNA for ABA 8'-hydroxylase (CYP707A2 gene), cultivar Yueyou 7, mRNA HG764751.1GAGTCTGACCGATAGCGCCTGCATGCTCATGTTGCCAGTCTTGCCACCAGTACCCGTTCCAGTGTGAGGAGCCGGAACTGACCT
ACGCCATTTTGTAAACACCCGGTAGAAGAGGAATAAGGACTCCCGGAAGTGTGCCAAGTACCAAGGTTTCAACTTCGGGTACGCAG
TCTACGAGACCAGTAAG**Plasmid 8: cDNA insert***Arachis hypogaea* cultivar fuhua 8 glutamate dehydrogenase 1 (GDH1) mRNA, KT933119.1ACTGGTCTCGTAGACTGCGTACCCGGTCAGGACTCATCGCTACGTTAGCGTCTCTGAGGCGCGCTATTCTACAATCTTAAAAAC
CCCTGTCAACCCCTTAAATTTGCTTTTAAAGACAATGATTTGCGCTTCTTTCTGATTTCTTCTTGGGGAGAAGAAAACCCGTGGGCTG
ACGTTGCTGCGGGGCGCACTTTACAAGCCTTTGCCTTACAGTTCAACGCCCTATCGGTCAGGACTCATAAGGGC**Plasmid 9: cDNA insert.***Arachis hypogaea* strain E2-4-83-12 delta-12 fatty acid desaturase (FAD2B) gene, JN544190.1TGAGTCTGACCGATAGCCTGCCTAAACCTTCTTGAAGTAGTGGCGGCGGTCGTTTTCCGGTACTGTCTGCTGGAAAATGTCCG
TCCAGAAAATCCCGCTCCATTACGTCCTGGTGAACATCACCCCGCAGATAACCTCCATCGGGTTGCACTTCAAAAAGCTCGGCAA
CCTTACGGCCTGCTTGACGCTCATTTTCATGTTTTCCCGCTTCTGTAGCGATGAGTCTGACCGGGTACGCAGTCTACGAGACC
AGTAAGGG**Plasmid 10 cDNA Insert.***Arachis hypogaea* cultivar fuhua 8 glutamate dehydrogenase 1 (GDH1) mRNA, Sequence ID: KT933119.1CTGGTCTCGTAGACTGCGTACCCGGTCAGGACTCATCGCTACGTTAGCGTCTCTGAGGCGCGCTATTCTACAATCTTAAAAAC
CCTGTCAACCCCTTAAATTTGCTTTTAAAGACAATGATTTGCGCTTCTTTCTGATTTCTTCTTGGGGAGAAGAAAACCCGTGGGCTGA
CGTTGCTGCGGGGCGCACTTTACAAGCCTTTGCCTTACAGTTCAACGCCCTATCGGTCAGGACTCATAAGGG**Plasmid 11: cDNA insert.***Arachis hypogaea* cultivar JL24 phenylalanine ammonia-lyase (PAL) mRNA, GU477587.1ACTGGTCTCGTAGACTGCGTACCCGACTAGTGAAGTATTACGCTTTCTTAAAGGGTGGCTGCTTCTAAGCCAACCTCCTAGCTGTCT
AAGCCTTCCCACATCGTTTCCCCTTAAACCATAACTTTGGGACCTTAGCTGACGGTCTGGGTTGTTTCCCTTTTACGACGGACGTTA
GCACCCGCGTGTGTCTCCATGCTCGGCACTTGTAGGATTCGGAGTTTGCATCGGTTTGGTAAGTCGGGATGACCCCTAGCCGA
AACAGTGTCTACCCCTACAGTGATACATGAGGCGCTACCTAAATAGCTATCGGTCAGGACTCATAAGGG**Plasmid 12: cDNA insert***Arachis hypogaea* cultivar JL24 phenylalanine ammonia-lyase (PAL) mRNA, ID: GU477587.1TGGTCTCGTAGACTGCGTACCCGACTAGTGAAGTATTACGCTTTCTTAAAGGGTGGCTGCTTCTAAGCCAACCTCCTAGCTGTCTAA
GCCTTCCCACATCGTTTCCCCTTAAACCATAACTTTGGGACCTTAGCTGACGGTCTGGGTTGTTTCCCTTTTACGACGGACGTTAGC
ACCCGCGTGTGTCTCCATGCTCGGCACTTGTAGGATTCGGAGTTTGCATCGGTTTGGTAAGTCGGGATGACCCCTAGCCGAAA
CAGTGTCTACCCCTACAGTGATACATGAGGCGCTACCTAAATAGCTATCGGTCAGGACTCATAAGGG

Continued

Plasmid 13: cDNA insert*Arachis hypogaea* beta-ketoacyl-ACP synthetase II-like (KASII) mRNA, FJ358425.1

CTGGTCTCGTAGACTGCGTACCCGACTAACCCATGTGCAAGTGCCGTTACATGGAACCTTTCCCTCTTCGGCCTTCAAAGTTCTCA
 TTGAATATTTGCTACTACCACCAAGATCTGCACCGACGGCCGCTCCGCCCGGGCTCGCGCCCCAGGTTTTGCAGCGACCGCCGCGC
 CCTCTACTCATCGCGCATAGCCCTTGCCCCGACGGCCGGGTATAGGTCACGCGCTTCAGCGCCATCCATTTTCGGGGCTAGTTGAT
 TCGGCAGGTGAGTTGTTACACACTCCTTAGCGGATGTGCGTCAGGACTCATAAGGG

Plasmid 14: vector DNA insert

gnl|uv|U37573.1:2706-4083 Phagemid vector pBK-CMV (+/-homology)

GGGCGAAGTGCCGGGGCAGGATCTCTGTACATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGGCGGT
 GCATACGCTTGATCCGGCTACCTGCCATTTCGACCACCAAGCGAAACATCGCATCGAGC.

Plasmid 15:cDNA insert*Arachis hypogaea* profilin (Ara h 5) mRNA: AF059616.1

GGATTTTATTTAGGAGGTATTGGGAACGAATTGGAATGTAATAATATTGATTCATAGAGATCCAGAAGAAAAAGAATAATCTTC
 TACTTTGAGAATAATAAAAAAAGAAAAGTGTCAATTGGAACATGAAAACCGTGACTGACTGAATTAGTTCTCGTTATTTTTA
 GGAAGGAGTGGAGATTATCGAACGAAGGATCCAATTACTTCGAAAAGAATTGAACGAGGAGCCGTATGAGGTGAAAATCTCAT
 GTACGGTTCTGTAGAGTGGCAGTAAGGATGACTTATCTGTCAACTTTTCCACTATTA

Plasmid 16: cDNA insert*Arachis hypogaea* mitogen-activated protein kinase 2 mRNA, DQ068453.1

TAATAGTGGAAAAGTTGACAGATAAGTCATCCTTACTGCCACTCTACAGAACCGTACATGAGATTTTCACCTCATACGGCTCCTCGTT
 CAATCTTTGAAAGTAATTGGATCCTTCGTTGATAATCTCCACTCCTTCCCTAAAAATAACGAGAACTAATTCAGTCAGTCACGTTT
 TCATGTTCCAATTGAACACTTTTCTTTTTTTTATTATCTCAAAGTAGAAGATTATTCTTTTTTCTTCTGATCTCTATGAATCAATATTAT
 TACATTCCAATTCGTTCCCAATACCTCCTAAATAAAAATCCAAGGGC

Plasmid 17 cDNA insert*Arachis hypogaea* ethylene-responsive element binding factor 1 (ERF1) mRNA, JQ048930.1

GAGTCTGACCGAGAACGGCATTGATAGCGATGAGTCTGACCGACAACGGCATTGATAGCGATGAGTCTGACCGGGTACGC
 AGTCTACGAGACCAGTAA

Plasmid 18 cDNA insert*Arachis hypogaea* triacylglycerol lipase 1 mRNA, GU902981.1:

TGAGTCTGACCGACAACGGCATTGATAGCGATGAGTCTGACCGGGTACGCAGTCTACGAGACCAGTA

Plasmid 19 cDNA insert*Arachis hypogaea* mitogen-activated protein kinase 2 mRNA:

DQ068453.1GATTTTATTTAGGAGGTATTGGGAACGAATTGGAATGTAATAATATTGATTCATAGAGATCCAGAAGAAAAAGAAT
 AATCTTCTACTTTGAGAATAATAAAAAAAGAAAAGTGTCAATTGGAACATGAAAACCGTGACTGACTGAATTAGTTCTCGTTAT
 TTTTAGGGAAGGAGTGGAGATTATCGAACGAAGGATCCAATTACTTCGAAAAGAATTGAACGAGGAGCCGTATGAGGNGAAAAT
 CTCATGTACGGTCTGTACAGTGGCAGTAAGGATGACTTATCTGTCAACTTTTNCCTATTACAAGGGCNAATTTCGCGGCCNGT
 NAATCCAATTCGCC

Plasmid 20: cDNA insert*Arachis hypogaea* arachin Ahy-3 mRNA, AY722687.1

GTCCTGACCGAGAACGGCATTGATAGCGATGAGTCTGACCGACAACGGCATTGATAGCGATGAGTCTGACCGACAACGGCATTGA
 TAGCGATGAGTCTGACCGGGTACGCAGTCTACG

Plasmid 21: vector DNA insert

gnl|uv|J01636.1:1-7477 E.coli lactose operon with lacI, lacZ, lacY and lacA genes.

CGTCGTGACTGGGAAAACCTGGCGTTACCCAACTTAATCGCCTTGACGACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAG
 GCCCGACCGATCGCCCTTCCAACAGTTGCGCAGCCTATACGTACGGCAGTTAAGGTTTACACCTATA

Plasmid 22: Vector DNA insert

gnl|uv|J01749.1:1-4361-49 Cloning vector pBR322

TAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTCCAT
 CCATAGTTGCTGACTCCCCGTCGTGTAGATAACTACGATACNGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGA
 GACCCAGCTCACCGGCTCCAGATTTATCAGCAATAAACAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCTGCAACTTTATCC
 GCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAG

Continued**Plasmid 23: Vector DNA insert**

gnl|uv|U37573.1:2706-4083 Phagemid vector pBK-CMV

CTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCATTTCGACCACCAAG
CGAAACATCGCATCGAGCGAGCACNGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCT
CGCGCCAGCCGAACGTTCGCCAGGC

Plasmid 24: Vector DNA insert

gnl|uv|AF028837.1:1577-1628 Cloning vector pKILHIS-1

TGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCA
GGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCCTTGCGGCGAATGGGCNTGACCGCTTCTCGTGCTTTACGGTATCGCC
GCTCCCGATTTCGACGCGCATCGCCTTCTATCGCCTTCTTGACGAGTCTTCTGAATTATTAACGCTTACAATTTCTGATGCGGTATTT
TCTCCTTACGCATCTGTGCGGTATTTACACCCGCATCAGGTGGCACTTTTCGGGAAAATGTGCGCGGAACCCCTATTTGTTATTTTT
CTAAATACATTCAA

Plasmid 25: Vector DNA insert

gnl|uv|X65311.2:2262-2409 Cloning vector pGEM-7Zf(-)

GCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCT
ATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCCTTGCGGCGAATGGGCTGACCGCTTCTCGTGCTTTACGGTATC
GCCGCTCCCGATTTCGACGCGCATCGCCTTCTATCGCCTTCTTGACGAGTCTTCTGAATTATTAACGCTTACAATTTCTGATGCGGT
ATTTTCTCCTTACGCATCTGTGCGGTATTTACACCCGCATCAGGTGGCACTTTTCGGGAAAATGTGCGCGGAACCCCTATTTGTTTAT
TTTTCTAAATACATT

Plasmid 27: Vector DNA insert

gnl|uv|NGB00350.1:415-785 Invitrogen pZErO-2 vector multiple cloning site

TCGTCTGTTTGTGGATGTACAGAGTGATATTATTGACACGCCGGGCGACGGATGGTGATCCCCCTGGCCAGTGCACGTCTGCTGTC
AGATAAAGTCTCCCGTGAACCTTTACCCGGTGGTGCATATCGGGGATGAAAGCTGGCGCATGATGACCACCGATATGGCCAGTGTGCC
GGTCTCCGTTATCGGGGAAAGAGTGGCTGATCTCAGCCACCGCGAAAATGACATCAAAAAACGCCATTAACCTGATGTTCTGGGGAAT
ATAAATGTCAGGCATGAGATTATCAAAAAAGG

Plasmid 29: vector DNA insert

gnl|uv|U37573.1:2706-4083 Phagemid vector pBK-CMV

GGGCGAAGTGCCGGGGCAGGATCTCCTGTCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCT
GCATACGCTTGATCCGGCTACCTGCCATTTCGACCACCAAGCGAAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCT
TGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTTCGCCAGGCTCAAGGCGAGCATGCCCGACG
GCGAGGATCTCGTCTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTTCATCGACTGTG
GCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGCGGCGGAATGGGCTGAC
CGTTCCTC

greenhouse, field plots, and greenhouse raised beds, and treated them with stoichiometric macro-mineral nutrient solutions; and obtained consistent good results on the RNA enzyme activity of GDH-synthesized RNA since 1998 [2].

3.4. Functional Structure of the GDH-Synthesized RNA

Possibility that the repeated plus/plus and plus/minus sequence matches among the GDH-synthesized oligonucleotide RNAs (Table 2) could resemble nucleic acid genetic codes encouraged a discussion on the functional relationship between genetic codes and the repeats in the GDH-synthesized RNA. The cDNAs of GDH-synthesized RNA were inserted into plasmids and sequenced (Table 3). The cDNA insert of plasmid 1 (Figure 1) shared one plus/minus sequence match with the mRNA encoding oxalate oxidase of peanut (Table 2). The cDNA insert of plasmid 2 shared one plus/plus sequence match with the mRNA encoding arachin h5 (profilin). The cDNA insert of plasmid 3 shared one plus/plus

sequence match with the mRNA encoding ubiquitin-conjugating enzyme. The cDNA insert of plasmid 4 shared one plus/minus sequence match with the mRNA encoding peanut type 2 metallothionein. The cDNA insert of plasmid 5 shared one plus/minus sequence match with the mRNA encoding peanut cultivar Yueyou 7 ABA 8'-hydroxylase [48]. The cDNA insert of plasmid 7 shared one plus/plus sequence match with the mRNA encoding peanut cultivar fuhua 8 glutamate dehydrogenase 1. The cDNA insert of plasmid 9 shared one plus/minus sequence match with the mRNA encoding peanut strain E2-4-83-12 delta-12 fatty acid desaturase. The cDNA insert of plasmid 10 shared a plus/plus sequence match with the mRNA encoding peanut cultivar fuhua 8 glutamate dehydrogenase 1, the second copy of that silencing RNA synthesized by GDH. The cDNA insert of plasmid 11 shared a plus/plus sequence match with the mRNA encoding peanut cultivar JL24 phenylalanine ammonia-lyase. The cDNA insert of plasmid 13 shared three sequence matches, two of which were plus/plus with +2/+1 frame shifts, and one was minus/minus with -2/-3 frame shifts to the mRNA encoding peanut beta-ketoacyl-ACP synthase II. This is a complex three-point binding by the GDH-synthesized RNA for wider sequence coverage and total silencing of the mRNA encoding ketoacyl-ACP synthase. Therefore, each insert cDNA of GDH-synthesized RNA (nongenetic code-based RNA) is a unit sequence code for a specific mRNA (genetic code-based RNA).

The DNA insert of plasmid 14 shared sequence match with a section of gnl|uv|U37573.1 phagemid vector pBK-CMV, a component of TOPO TA cloning vector.

The cDNA insert of plasmid 15 shared a plus/plus sequence match with peanut Ara h5 mRNA, being the second copy of that RNA synthesized by GDH. These are evidence that the synthesis of RNA by GDH is reproducible although it is template-independent. The cDNA insert of plasmid 16 shared two plus/minus sequence matches with the mRNA encoding peanut mitogen-activated protein kinase 2 (DQ068453.1); and with the mRNA encoding peanut mitogen-activated protein kinase 3 (EU182580.2). Therefore, the same fragment of RNA synthesized by GDH shared homology with two different mRNAs. This is another example of the complex mechanism, may be involving silencing RNA mass action effect (organic chemical reaction) on mRNA silencing by GDH-synthesized RNA. The cDNA insert of plasmid 17 shared minus/minus sequence match with -1/-3 frame shift to the mRNA encoding *Arachis hypogaeae* thylene-responsive element binding factor 1 [49]. The cDNA insert of plasmid 18 shared plus/plus sequence match with the mRNA encoding *Arachis hypogaea* triacylglycerol lipase 1. The cDNA insert of plasmid 19 shared three plus/plus sequence matches with the mRNA encoding peanut mitogen-activated protein kinase 3 (EU182580.2); with the mRNA encoding peanut mitogen-activated protein kinase 2 (DQ068453.1); and with the mRNA encoding peanut mitogen activated protein kinase 1 (DQ068452.1). Plasmid 19 is different from plasmid 16. Therefore, GDH synthesized two different silencing RNA fragments that targeted different sites in the mRNAs encoding the mitogen-activated protein kinases. This

is another example of the complex mechanism involving reiterated RNA enzyme mass action effect on mRNA silencing. The cDNA insert of plasmid 20 shared two plus/plus sequence matches with the mRNA encoding arachin Ary-3.

The DNA insert of plasmid 21 shared sequence match with a section of gnl|uv|J01636.1 *E. coli* lactose operon lacI, lacZ, lacY and lacA genes, a component of PCR 4 TOPO TA cloning vector. The DNA insert of plasmid 22 shared sequence match with a section of gnl|uv|J01749.1 of pBR322, a component of PCR 4 TOPO TA cloning vector. The DNA insert of plasmid 23 shared sequence match with a section of gnl|uv|U37573.1 phagemid pBK-CMV. The DNA insert of plasmid24 shared sequence match with a section of gnl|uv|X65311.2 vector pGEM-7Zf(-), component of TOPO TA cloning vector. The DNA insert of plasmid25 shared sequence match with a section of gnl|uv|X65310.2 vector pGEM-7Zf(+), component of TOPO TA cloning vector. The DNA insert of plasmid 27 shared sequence match with a section of gnl|uv|NGB00350.1 Invitrogen pZErO-2 vector multiple cloning site, component of TOPO TA vector. The DNA insert of plasmid 29 shared sequence match with a section of gnl|uv|U37573.1 phagemid vector pBK-CMV, a component of TOPO TA cloning vector. Cloning vector DNA inserts 14, 23, and 29 are different lengths from the same pBK-CMV section of TOPO TA vector.

Therefore, the GDH-synthesized RNA oligonucleotide units (**Table 3**) represent the biological codes for recognition of specific mRNA targets in total RNA, the RNA enzyme activity (**Figure 2**) being the biochemical mechanism for eliminating the undesired mRNAs.

3.5. Biotechnological Applications of GDH-Synthesized RNA

In vitro demonstration of the RNA enzyme activity of GDH-synthesized RNA reveals a smart approach to save time, space and effort on basic research experimentations with plants because preliminary surveys of the responses of plant metabolism to the environment could be conducted at reduced scales in growth chambers, greenhouse, and field plots specifically to collect sufficient tissues for total RNA and GDH purifications; the phenotypic responses of the plant to mineral nutrients, biochemical regulators, agro-chemicals and other xenobiotics being conducted and interpreted in biochemical cross-over reactions as in **Figure 2**. The more severe the degradation of total RNA is, the more suppressed will the biochemical pathways be, and accordingly the more pronounced the phenotypic characteristics of the plant would be. The GDH-synthesized RNA enzyme could be labeled to differentiate its identity from total RNA moieties in the cross-over reactions in **Figure 2**.

Dismantling of the structural constraints imposed on RNA by genetic code liberated RNA to become an enzyme with specificity to degrade unwanted transcripts not on base-pairing as in double stranded siRNA, but on the basis of homologous sequence alignment recognition (**Figure 2**). This nascent biochemical knowledge describing the natural RNA enzyme activity of the ubiquitous GDH synthesized RNA is perhaps of utmost importance in basic enzymology and mo-

lecular biology research because the activity readily liquefies homologous transcripts, not complementary transcripts. The discovery of the RNA silencing and splicing processes [10] [43] [50] [51] unleashed a new biology that spontaneously elevated the realms of human ingenious imagination in the R & D sciences of medicine and agriculture. Over the past decade, the genetic code-based siRNA mechanism has delayed some of the anticipated technological breakthroughs. Biochemical experimentations with plants [1]-[9] [25] [26] [29] [45] [46] however continued to intuitively suggest the existence of an extensive and generalized metabolic network control system at the RNA level but of a somewhat distinct chemical apparatus from the genetic code-based siRNA system [10] [43] [50] [51]. The RNA logic of the generalized chemical silencing apparatus is more vividly illustrated (**Figure 2**). Therefore, the nongenetic code-based RNA enzyme may provide wider molecular approaches for controlling the population of undesired transcripts in plant systems.

GDH also synthesizes unit oligonucleotides that silence its own encoded mRNAs (**Table 3**), a perfect feedback strategy for controlling and for automatically shutting off the RNA enzyme concentration when the environmental conditions are conducive for the rapid growth of the plant. Control of the abundance of transcript populations in plant cells could henceforth be accomplished simply and specifically by applying stoichiometric mixes of mineral nutrient, other agrichemical, N-carboxymethyl chitosan, or biochemical regulator solutions to the plant without the need to re-construct any genes. This is a considerable simplification of scientific research thought in a majority of plant enzymology and molecular biology investigations.

The cDNAs of GDH-synthesized RNA are the Northern blot tools for unraveling the molecular mechanisms underlying the multitude of metabolic networks and biochemical regulations in the cell. Since the GDH-synthesized RNA matches transcript sequences in repeated plus/plus, minus/minus, and/or plus/minus homologous orientations [4] [7] [8] [9] the binding of cDNA strands as labeled probes to transcripts is fail-proof because both strands could potentially bind to the same transcript in Northern hybridization thereby enhancing efficiency of detection of Northern bands. Synthesis of RNA enzyme by GDH is therefore an alternative biotechnology to algorithm design [52] [53] of siRNA and Northern probes. GDH synthesized RNA is however for experimentation on the coordination of biochemical pathways and of metabolic networks. There are yet no algorithm-designed hybridization probe and siRNA for the study of integration/discrimination regulation of biochemical pathways. The frame shifts in the structure of GDH-synthesized RNAs enlarges the span of mRNA/RNA enzyme interaction for improvement of mRNA destruction.

In the application of algorithm-designed siRNAs for the silencing of transcripts, it has been reported that siRNA efficacy is limited by target transcript abundance and turnover rate [54] [55]. The biological phenomenon of transcript turnover rate is further exacerbated by the transcriptome profiling of total tran-

script extracts from experimental organisms in comparison with an untreated organism as control. Such an untreated control is in its right an experimental treatment, but with unknown chemical environmental conditions. There should be a second control organism with known chemical environmental condition housing the specific control target transcripts. In the induction of GDH synthesized RNA enzyme experimentation, in addition to the control crop, there is a second control crop housing the control GDH [7] [26]. Whereas the control crop's transcripts display many of the turnover rates and abundance observed for the experimental organisms, the transcripts of the control crop that is housing the control GDH are less abundant, displaying a diminished turnover rate pattern that is visibly different from those of the experimental organisms, thereby making for unequivocal and an accurate interpretation of Northern blot and transcriptome results from siRNA profiling experimentations [8] [9]. These considerations emphasize the importance for the inclusion of GDH-synthesized RNA enzyme assays as necessary controls in plant systems molecular biology research projects.

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Conflicts of Interest

The authors declare no financial competing interests.

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