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Total RNA Degradation *in Vitro* and *in Vivo* by Glutamate Dehydrogenase-Synthesized RNA Enzyme: Biotechnological Applications

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Abstract

Glutamate dehydrogenase regulates crop development, growth, and biomass yield through its synthesis of non-genetic code-based RNA. Understanding the mechanism of GDH-synthesized RNA enzyme would enhance the agriculture innovation capacity of the more than a billion urban gardeners, smallholder, and limited resources indigenous farmers. Different metabolic variants were prepared by treating peanut growing on healthy soil with stoichiometric mixes of mineral salt solutions. Peanut GDH charge isomers were purified to homogeneity by electrophoresis, and made to synthesize RNA enzyme. Peanut total RNA was 5'-end labeled with $[\gamma\text{-}^{32}P]ATP$ and made to react as substrate *in vitro* with GDH-synthesized RNA from another metabolic variant of peanut. Agarose, and polyacrylamide gel electrophoresis of the reaction products showed that tRNA, rRNA, and most of the mRNAs were degraded to mononucleotides, but total RNAs that were not mixed with GDH-synthesized RNAs were not degraded. When the non-homologous sequence sections of the GDH-synthesized RNA were clipped out, the homologous sections failed to produce Northern bands with peanut total RNA. Therefore, the non-homologous sequence sections served to identify, position, and align the GDH-synthesized RNA to its target total RNA site independent of genetic code; the degradation of total RNA being via non-canonical base alignments in the enzyme-substrate complex, followed by electromagnetic destruction of the total RNA, the less stable of the two kinds of RNA. This is the science-based corner stone that buttresses the crop production efforts of limited resources farmers because GDH-synthesized RNAs quickly degrade superfluous total RNA of the crop in response to the soil mineral nutrient deficiencies thereby minimizing wastage of metabolic energy in the synthesis of unnecessary protein enzymes while optimizing biomass metabolism, crop

growth, and maximum crop yields. *In vitro* hydrolysis of total RNA by GDH-synthesized RNA is the game changing, prototype, R&D methods for cleansing sick total RNA from cells, tissues, and whole organisms.

Keywords

1. Introduction

Crop glutamate dehydrogenase (GDH, EC 1.4.1.2) synthesizes RNA independent of template as it isomerizes in response to nucleophiles and electrophiles including intermediary metabolites, xenobiotics, pesticides, mineral nutrients, N-(Carboxymethyl) chitosan, methionine sulphoximine, auxins, toxic metal ions, and nucleotides [1] [2] [3] [4]. The RNA synthetic activity of GDH and its coordination of biochemical pathways were initially described as signal integration and discrimination phenomena [4]. Later, comparison between the nucleotide sequences of numerous RNAs synthesized by GDH and crop yields on the one hand, and with their responses to stoichiometric mixes of mineral salt treatments on the other hand revealed the ribonuclease activities of the RNAs [5] [6]. The enzymic property of GDH-synthesized RNA (non-genetic code-based RNA) is due in parts to the fact that it is more thermostable than total RNA [7]. The enzymic mechanism may involve non-canonical electrostatic base pairing chemical reactions [8] [9] [10] [11] [12]. Hereunder, we begin to present the machinery and mechanisms of total RNA degradation by GDH-synthesized RNA, and of the possible R&D applications.

Transcriptomic analyses of plants that were exposed to differential mineral nutrient conditions revealed the identity of several metabolic pathways and many genes that were the targets of the actions of the mineral nutrients [13], but the chemical/molecular mechanisms of the differential responses were not presented. RNA degradation activities are prevalent in many genomes; most ubiquitous mechanisms being those of endonucleases, 5' exonucleases, and 3' exonucleases [14] [15]. DNases and RNases are not controlled by agronomic abiotic stress factors. In the cytoplasm, double-stranded RNAs (miRNA, siRNA, shRNA etc.) are activated by RISH and RITS protein complexes to direct site-specific degradation of complementary RNAs [16] [17]. Gene silencing by short RNA duplexes has been demonstrated to repress protein expression [16] [18] [19] in plants, mammals, and invertebrates. RNA interference phenomenon was first recognized as antiviral mechanism that protects organisms from RNA viruses [20]. But gene silencing by RNA interference mechanism is the degradation of genetic code-based RNA by genetic code-based RNA; therefore, it has limitations in its utility in smallholder crop production agriculture.

Glutamate dehydrogenase being sensitive to abiotic and biotic redox environments (drought, light, agronomic practice, extreme temperatures, extreme pH values, variable salinity, variable mineral nutrient compositions and concentrations, variable soil organic carbon contents, pathogen infection etc.), it dynamically links plant growth and development to soil health and mineral nutrient composition and concentration [21] [22]. When soil health, mineral concentration and composition are adequate, crop's GDH activity responds favorably, and crop yield doubles and optimizes [22] [23]; but when the soil is degraded, then crop growth, development and productivity usually decline [24] [25] [26] [27]. Healthy soil begets healthy crops, which in turn beget healthy human nutrition. Nutrient budgeting to assure optimal application of fertilizers to the soil has not provided acceptable guidelines for the analysis of agronomic uncertainties [28]. Integrated nutrient management for precision fertilization of soil is complicated for limited resource farmers to implement in the light of nutrient loss and resultant agroecosystem degradation.

Genetic code-based RNAs (rRNA, tRNA, and mRNA) catalyze the biosynthesis of proteins, the hallmarks that enable plants to grow, develop and accumulate biomass feed and food stuff. Differential availability of mRNAs charged with ribosomes is one of the factors that regulate the rate of protein biosynthesis [29]. Therefore, the regulation of the abundance of genetic code-based RNAs by GDH-synthesized RNA enzyme is very important for optimal agricultural productivity. Plants naturally grow in degraded soils with mineral nutrient limitations [30]. Close to 800 million people in the world or 78% of the world's indigenous poor peoples—live in rural areas and rely on crop production on degraded soil to put food on their dining tables [31]. More than 500 million smallholder farmers manage the majority of the world's agricultural land and produce most of the world's food, phyto-medicines, and feed. Farming in the city backyard community gardens account for 15% of the world's fresh, and nutritious food production (https://www.farmers.gov/media/blog/2018/11/06/farming-city). Farms of less than 1 hectare account for 72% of all farms. In contrast, only 1% of all farms in the world are larger than 50 hectares [32]. With urban population and hunger rising up to 50% [33], the summation of urban gardeners, city farmers, and 800 million rural farmers indicates that more than 1 billion people are cultivating the land to produce their fresh, healthy food, create jobs, and generate income. Limited resource farmers lack the financial power to purchase sufficient fertilizers, pesticides, irrigation technology (http://129.114.16.46/AgroNew/index.php), and agricultural machinery in support of their crop production efforts. Despite the fact that the world depends on subsistence, limited resource, and smallholder famers for sustainable consistent production of fresh, healthy food and feed crops, there has been no science-proven technology that buttresses and encourages the agricultural efforts of smallholder crop producers. However, understanding the machinery and chemical mechanism of the degradation of genetic code-based RNA by nongenetic code-based RNA (GDH-synthesized RNA) may

encourage the construction of new strategies that leverage on GDH activity and thus enhance the agriculture innovation capacity of smallholder farmers.

GDH is a machinery that differentially controls total RNA concentrations according to the biomass metabolic needs of the crop [1] [22]. GDH is multi-isoenzymic. It is purified as polypeptides by chromatography [34] [35] [36] or as intact, active, undegraded isoenzymes by electrophoresis [37]; the template-independent RNA synthetic activity being the exclusive function of the active undegraded, non-denatured isoenzymes [1] [22]. We had provided qualitative evidence for the degradation of genetic code-based RNA by GDH-synthesized RNA [7]. Hereunder we describe the degradation of total RNA *in vitro* and *in vivo* as the mechanisms by which GDH isoenzymes control growth, differentiation, and nutritious biomass accumulation in peanut cultivated in the abiotic stress environment of mineral nutrient-limited soil, which resembles and mimics the limited resources farmer's farm plot.

2. Materials and Methods

2.1. Treatment of Peanuts with Stoichiometric Mixes of Mineral Salt Solutions

Peanut (*Arachis hypogaea* L. Cv. Virginia) seeds were planted in $120 \times 120 \times 30$ cm (width × length × depth) boxes (raised beds), each filled with healthy soil prepared by mixing two bags (18 kg) of top soil (Landscapers Pride, New Waverly, Texas, USA) and three bags (2.8 cu ft.) of professional growing mix (Sungro Horticulture, Bellevue, Washington, USA). Each raised bed was set up on level ground in the field on weed-blocking plastic mat, in the University farm, Waller County, Texas, USA. About 30 seeds were planted in each of 50 raised beds in late May, 2014. From the 50 beds, 22 were selected in which the seed germination was 100%, and the peanut seedlings were growing vigorously at about the same rate. The first 2 beds were left as the untreated control. Treatments were made in replicate as described in Table 1 [39]. The applied mineral salt compositions were based on stoichiometric combinations to mimic the binomial subunit polypeptide compositions of the GDH isoenzymes [38] and to interact with target molecules in peanut in molar ratios. All the boxes were watered equally every other day. Chitosan and mineral solutions (5 L per bed) were applied three times: first at 3 weeks after seed germination, second at flowering, and the last was at post-flowering. When the leaves turned yellow and dry (peanut maturity), pods were harvested, weighed per box and immediately shelled by hand, and the kernels (seeds) were stored at −30˚C. The replicate seed harvests were not combined but stored separately.

2.2. Purification of GDH

GDH isoenzymes were extracted from peanut seeds (30 g) with 0.1 M Tris-HCl buffer containing RNase A [1] [22]. The seeds were from the control or mineral salts-treated raised beds. After ammonium sulfate salting out step, and dialysis

Table 1. Some Stoichiometric mixes of mineral salts that produced metabolic variants of peanut through the activities of GDH-synthesized RNA enzyme. The peanuts growing in greenhouse raised beds were fertilized with the solutions of the stoichiometric mixes of mineral salts, and of N-carboxymethyl chitosan.

Treatments
N (1 L 25 mM NH_4Cl)
N + P + K + S (1 L 25 mM NH_4Cl, 20 mM Na_3PO_4, 4 mM KCl, plus 50 mM Na_2SO_4)
K (1 L 4 mM KCl)
S (1 L 50 mM Na_2SO_4)
P + S (1 L 20 mM Na_3PO_4, plus 50 mM Na_2SO_4)
P (1 L 20 mM Na_3PO_4)
N + S (1 L 25 mM NH_4Cl plus 50 mM Na_2SO_4)
N + P + K (1 L 25 mM NH_4Cl, 20 mM Na_3PO_4, plus 4 mM KCl)
P + K (1 L 20 mM Na_3PO_4, plus 4 mM KCl)
Control (untreated)
0.1% N-Carboxymethyl Chitosan solution.

to remove the ammonium sulfate, the crude extract was subjected to Rotofor (Bio-Rad) isoelectric focusing [3]. The pI values of the fractions were determined, followed by dialysis to remove the ampholyte (Bio-Rad's Bio-Lyte 3/10). The Rotofor fractions (0.2 mL) were loaded onto duplicate native 7.5% polyacrylamide gels and electrophoresed (Bio-Rad protean II xi cell). After native gel electrophoresis, one gel was stained with the phenazine methosulfate-glutamate-NAD^+-tetrazolium bromide solution [40] in order to locate the positions of the GDH isoenzymes. GDH isoenzyme distribution pattern in the gel landscape was photo-documented. Using the stained gel as guide/template on a lightbox, the location of the GDH isoenzymes was excised from the duplicate electrophoresed gel [37] [41]. The GDH isoenzymes were electro-eluted in 0.05M Trizma solution from the excised piece of gel using Bio-Rad whole gel eluter at sub-zero degree temperature in a freezer [41]. The fractions from the whole gel eluter were not combined. The cryoelectrophoresis was done many times in order to purify enough isoenzymes for the synthesis of RNA. All the batches of purified GDH charge isomers per treated peanut had identical distribution of GDH subunit polypeptides.

2.3. Synthesis of RNA Enzyme

RNA synthetic activity of the GDH isoenzymes [18] was assayed in the amination substrate solutions of 0.1 M Tris-HCl buffer (pH 8.0) containing the four NTPs (0.6 mM each), $CaCl_2$ (3.5 mM), NH_4Cl (0.875 mM), α-ketoglutarate (10.0 mM), NADH (0.225 mM), 5 Units RNase inhibitor, 1 Unit of DNase-1, and 5 μg of actinomycin D. Reaction was started by adding 0.2 mL of whole gel-eluted GDH charge isomers containing 9 - 20 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) removal of the proteins [42]. The RNA (enzyme) was precipitated with ethanol, and dissolved in minimum volume of molecular biology quality water; and stored at −20°C before use. RNA enzyme yield and quality were determined by photometry and by agarose gel electrophoresis using RNA molecular weight markers and peanut total RNA as standards. The agarose gel was stained with ethidium bromide, and the RNA yield/distribution pattern was photo-documented. Assays were carried out in duplicate (from the duplicate seeds harvested) to verify the reproducibility of the results. The GDH isoenzyme patterns for the duplicate seed harvest per experimental treatment of peanut were similar. Replicate seed yields that gave similar/identical GDH patterns per experimental treatment were then combined for other downstream analyses. GDH purification and GDH synthesis of RNA were completed within 3 weeks after peanut seeds were harvested.

2.4. Total RNA

Total RNA was extracted from peanut seeds (15 g) harvested from the control or mineral salt-treated boxes using the acidic phenol/chloroform (pH 4.5) method [42].

2.5. End-Labeling of Total RNA as Substrate

Peanut total RNA (~5 μg) was dephosphorylated with calf intestinal alkaline phosphatase; and labeled with 1 μL of [γ^{32}-P] ATP (700 Ci/mmol) (ICN Biochemicals, OH, USA) using I μL of T4 polynucleotide kinase (Kinase-Max kit, Ambion, Austin, TX, USA) (10 units/μL) and 2 μL 10X Kinase buffer added to the reaction cocktail to bring the total volume to 20 μl. Reaction was incubated at 37°C for 1 h, and stopped by adding EDTA to make the reaction 1 mM, then heated to 95°C for 3 min. The labeled RNA was not further purified.

2.6. *In Vitro* Hydrolysis of Total RNA by GDH-Synthesized RNAs

The RNAs (enzymes) synthesized by the GDH charge isomers were arranged into 7 groups (very acid, acid, mildly acid, neutral, mildly alkaline, alkaline, and very alkaline) in the ascending order of their pI values. Labeled total RNA (substrate) (2.5 μg) of a different treatment of peanut was added to 20 μg of GDH-synthesized RNA (enzyme) from another treatment of peanut, and the total volume was brought to 49 μL with 0.1 M Tris-HCl buffer solution pH 8. Ribonucleoside triphosphate (riboNTP) mix (0.6 mM each riboNTP) 1 μL was added to the reaction cocktail on ice. Each of the 7 groups of GDH-synthesized RNA enzyme per experimental peanut was used for the hydrolysis. The reaction was thermo-cycled as described before [7]: pre-heat (96°C, 30 sec), then 40 cycles (unless otherwise stated) of cool (5°C, 1 min), and warm (37°C, 2 min). At the end of thermo-cycling, the reaction was held at 5°C. Two controls with-

out GDH-synthesized RNA were prepared, each contained 2.5 µg of labeled total RNA and 1 µL of the riboNTP mix in a final volume made up to 50 µL with 0.1 M Tris-HCl buffer solution pH 8.0. One of the controls was thermo-cycled with the experimental reactions, the other was not thermo-cycled but it was left on ice.

Two approaches were developed for fractionating the degradation products. The first was by agarose gel electrophoresis, and the second was by polyacrylamide gel electrophoresis. Any non-degraded total RNA, degraded total RNA, and excess unreacted ^{32}P-ATP were revealed by a brief (30 - 45 min, 70 volts, TAE buffer) agarose gel (3.0%) electrophoresis of 5 µL of each hydrolysis reaction solution. The electrophoresed agarose gel was electro-trans-blotted (Bio-Rad semi-dry sub-cell) onto BrightStar Plus Nylon membrane (Applied Biosystems, Foster City, CA, USA) as described before [23], and the membrane was autoradiographed.

Any undegraded total RNA, and degraded total RNA were converting to RNA:DNA hybrids followed by polyacrylamide gel electrophoresis. To the hydrolyzed RNA solution (15.0 µL), the following Display Systems Biotech, Vista, CA reagents were added: anchored primer (5' T$_n$V 12.5 µM) 2.0 µL, 10X cDNA buffer 1 (2.5 µL), dNTP mix (5 mM each) 5.0 µL, displayTHERMO-RT (100 U/µL) 1.0 µL. The reaction was incubated for 2 h at 42˚C. Then the tube was placed on ice. Polyacrylamide gel (10%) was made from 29:1 acrylamide:bisacrylamide mixture in 1XTBE and 7 M urea [43]. The first strand reaction products (10 µL) was loaded in 1X glycerol loading buffer [43] and electrophoresed in a vertical Bio-Rad Protean II ix cell, at room temperature, 1× TBE buffer and at constant 20 mA for 45 min. The electrophoresed gel was dried (Bio-Rad gel dryer), and the gel was autoradiographed.

2.7. Preparation of cDNA of GDH-Synthesized RNA

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 [38] 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 (Novagen, Madison, WI). The insert cDNA was sequenced with T3 and T7 primers by Genemed Synthesis, Inc. (South San Francisco, CA, USA), and Functional Biosciences, Inc. (Madison, WI, USA). To identify the GDH-synthesized RNAs that were homologous to genetic code-based RNAs (mRNAs, tRNAs, and rRNAs) the cDNA sequences were used as queries to search the NCBI nucleotide-nucleotide

(excluding ESTs) BLAST (blastn), and non-redundant protein translation (blastx) databases. Complementary DNAs that displayed the highest alignment scores with genetic code-based RNAs of the correct molecular weights were selected as Northern probes.

2.8. Labeling the cDNA as Northern Probe

The cDNAs that were used as Northern probes were those homologous to mRNAs encoding the enzymes of primary metabolism (starch synthase, cytochrome P450 reductase, acetyl coenzyme A carboxylase, flavonoid biosynthetic enzyme etc) and of the rRNAs of peanut [38]. For the labeling of the cDNA probes, cDNA inserts were amplified by PCR from the corresponding plasmids (15 ng) using M13 forward and M13 reverse primers (2 μM each), [^{32}P]-dATP (6000 Ci/mmol, 20 mCi/mL), dCTP/dGTP/TTP mix 50 mM, (2 μL), and Taq polymerase (1U), in a final volume of 50 μL. Amplification was according to Display Systems Biotech (Vista, CA, USA) 'touch-down' PCR procedure (denature: 94°C, 1 min. For the first 10 cycles: 94°C, 30 sec; anneal: 60°C, 30 sec for the first cycle, then reduced the temperature 0.5°C each cycle until an annealing temperature of 55°C was reached after 10 cycles; extension: 72°C, 1 min. Continued another 25 cycles with 94°C, 30 sec; 55°C, 30 sec; 72°C, 1 min; final extension 72°C, 5 min).

2.9. Chemical Synthesis and Labeling of Oligonucleotide Probes

The homologous section of each GDH-synthesized RNA was clipped out of the non-homologous 3'-, and 5'-termini, and the complementary strand of the oligonucleotide was chemically synthesized by Sigma Life Science, The Woodlands, Texas, USA. The oligonucleotide (1.0 pmol) was labeled with 1 μL of [γ^{32}P] ATP (7000 Ci/mmol) (ICN Biochemicals, OH, USA) using I μL of T4 polynucleotide kinase (Kinase-Max kit, Ambion, Austin, TX, USA) (10 units/μL) and 2 μL 10× Kinase buffer added to the reaction cocktail to bring the total volume to 20 μl with molecular biology quality water. Reaction was incubated at 37°C for 1 h, and stopped by adding EDTA to make the reaction 1 mM, then heated to 95°C for 3 min. Excess unreacted [^{32}P] ATP was removed by chromatography of the reacted solution through Ambion NucAway spin column.

2.10. Northern Blots

Equal amounts (15 μg) of total RNA from the control and mineral salt-treated peanuts were loaded, briefly electrophoresed on 2% agarose gels, stained with ethidium bromide, and photographed to verify RNA quality. RNA was electro-transferred from the electrophoresed gel onto Brightstar-Plus nylon membrane (Applied Biosystems, Foster City, CA, USA) as described before [44].

Nylon membranes with immobilized RNA were prehybridized with UL-TRAhyb buffer and hybridized with ^{32}P-labeled cDNA inserts or the corresponding Sigma-synthesized oligonucleotides as probes overnight at 68°C as de-

scribed before [23] [44] Solutions of labeled cDNA were first heated in boiling water bath for 10 min before adding to the prehybridized membrane. After hybridization, the membranes were washed (20 min, 68˚C) with NorthernMax (Applied Biosystems, Foster City, CA, USA) low stringency wash solution followed by NorthernMax high stringency wash solution (15 min, 60˚C). The membrane was autoradiographed by exposure to X-ray film within intensifying screens at −80˚C. Northern band intensities were digitalized using UN-SCAN-IT gel digitalizing software (Silk Scientific, Inc., Orem, Utah).

3. Results

### 3.1. *In Vitro* Degradation of the Total RNA of KCl-Treated Peanut by GDH-Synthesized RNA of P + K-Treated Peanut

The RNA enzymes synthesized by all the GDH charge isomers (a) very acid (pI 4.5 ± 0.2); (b) acid (pI 5.0 ± 0.2); (c) mildly acid (pI 6.0 ± 0.4); (d) neutral (pI 7.0 ± 0.4); (e) mildly alkaline (pI 8.0 ± 0.3); (f) alkaline (pI 8.4 ± 0.2); (g) very alkaline (pI 8.6 ± 0.1) of the P + K-treated peanut hydrolyzed all the low molecular weight constituents (tRNA, 5S, 16S and 28S rRNAs, and many mRNAs) of the total RNA (substrate) of KCl-treated peanut to virtually mononucleotides compared with the total RNA samples that were not treated with the GDH-synthesized RNAs (Figure 1(A)). This is the only RNA enzyme that degrades total RNA *in vitro*. The RNAs synthesized by the acid charge isomers of GDH were more efficient than the others in the degradation of total RNA judging from the scanty undegraded total RNA at the origin of the agarose gel well. When RNA is relieved of coding function, it becomes a fully-fledged enzyme [7].

Much of the full length RNA:DNA hybrids of total RNA (substrate) were also stuck at the origin (Figure 2(A)); whilst the degradation intermediates (bands 2, 3, 4, 5, and 6) were also captured by the polyacrylamide gel (Figure 2(A)). With the undegraded total RNA as the base line (Figure 2(A)), digital quantitation of the degradation products of the total RNA showed that the RNA enzyme synthesized by the acid GDH isomers was about two to five folds more active than the enzyme synthesized by the neutral charge isomers of GDH. However, the RNA enzymes synthesized by the neutral (pI 7.0 ± 0.4), mildly alkaline (pI 8.0 ± 0.1), and alkaline (pI 8.4 ± 0.2) charge isomers of GDH also degraded total RNA to different extents. Agarose gels did not capture the intermediate degradation products (Figure 1). GDH-synthesized RNA is the only enzyme that degrades total RNA *in vitro*. The presence of intermediate degradation bands of total RNA suggested that an enzyme-substrate complex was formed in the degradation reaction.

3.2. *In Vitro* Degradation of Total RNA of Control Peanut by GDH-Synthesized RNA of P + K-Treated Peanut

The RNA enzymes synthesized by (a) very acid (pI 4.5 ± 0.2); (b) acid (pI 5.0 ± 0.2); (c) mildly acid (pI 6.0 ± 0.3); (e) mildly alkaline (pI 8.0 ± 0.3); (f) alkaline

(pI 8.4 ± 0.2); (g) very alkaline (pI 8.6 ± 0.2) charge isomers of the GDH of the P + K-treated peanut hydrolyzed all the low molecular weight constituents (tRNA, 5S, 16S and 28S rRNAs, and some mRNAs) of the total RNA (substrate) of control peanut to virtually mononucleotides compared with the total RNA samples that were not treated with the GDH-synthesized RNAs (Figure 1(B)). The most active RNA enzymes were those synthesized by the very acid and acid charge isomers. This is the only RNA enzyme that degrades total RNA *in vitro*. The RNA enzymes synthesized by the mildly acid (pI 6.0 ± 0.4), and neutral (pI 7.0 ± 0.4) charge isomers of GDH were less active in the degradation of total RNA judging from the similarity of their undegraded total RNA with those of
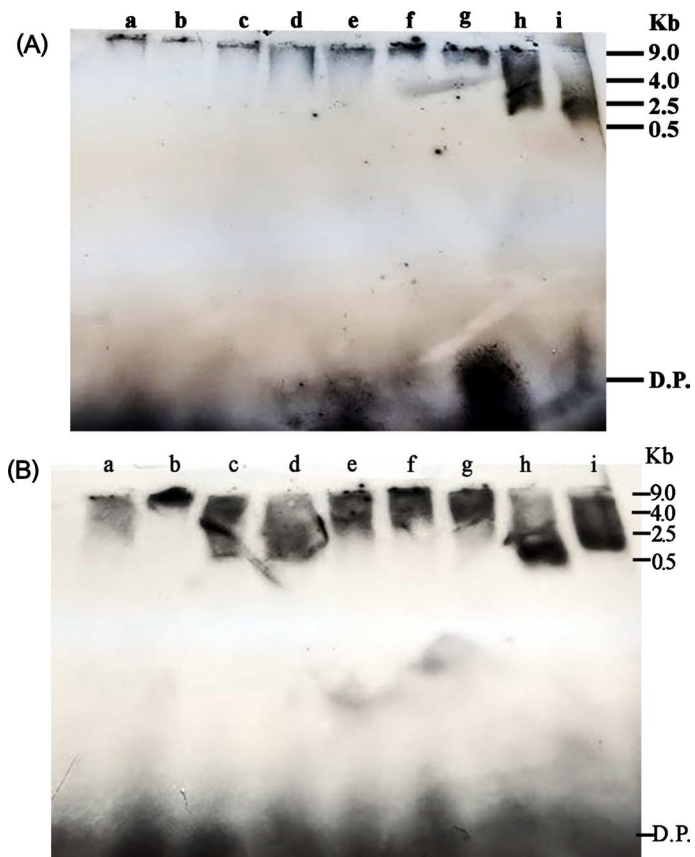
Figure 1. *In vitro* total RNA degradation products (agarose gel electrophoresis). GDH charge isomers in micro tubes (a) very acidic (pI 4.5); (b) acidic (pI 5.0); (c) mildly acidic (pI 6.0); (d) neutral (pI 7.0); (e) mildly alkaline (pI 8.0); (f) alkaline (pI 8.4); (g) very alkaline (pI 8.6) were purified by electrophoresis from P + K-treated peanut, and made to synthesize RNA enzyme. Peanut total RNA was 5' end-labeled with [γ^{32}P]-ATP. Labeled total RNA (20 µg) of KCl-treated peanut (A); and control peanut (B) was added to each micro tube containing RNA enzyme synthesized by each GDH charge isomer in 0.05 M Tris-HCl buffer solution pH 8.0. Micro-tubes (h), and (i) contained 20 µg of labelled total RNA in 0.05 M Tris-HCl buffer solution pH 8.0 but no GDH-synthesized RNA enzyme. Tubes (a), (b), (c), (d), (e), (f), (g), and (h) were thermo-cycled, but tube (i) was left on ice. After the reaction, 5 µL of each reaction was briefly electrophoresed through 3% agarose gel. The electrophoresed gel was trans-blotted to nylon membrane, and the membrane was autoradiographed. DP means degradation products.
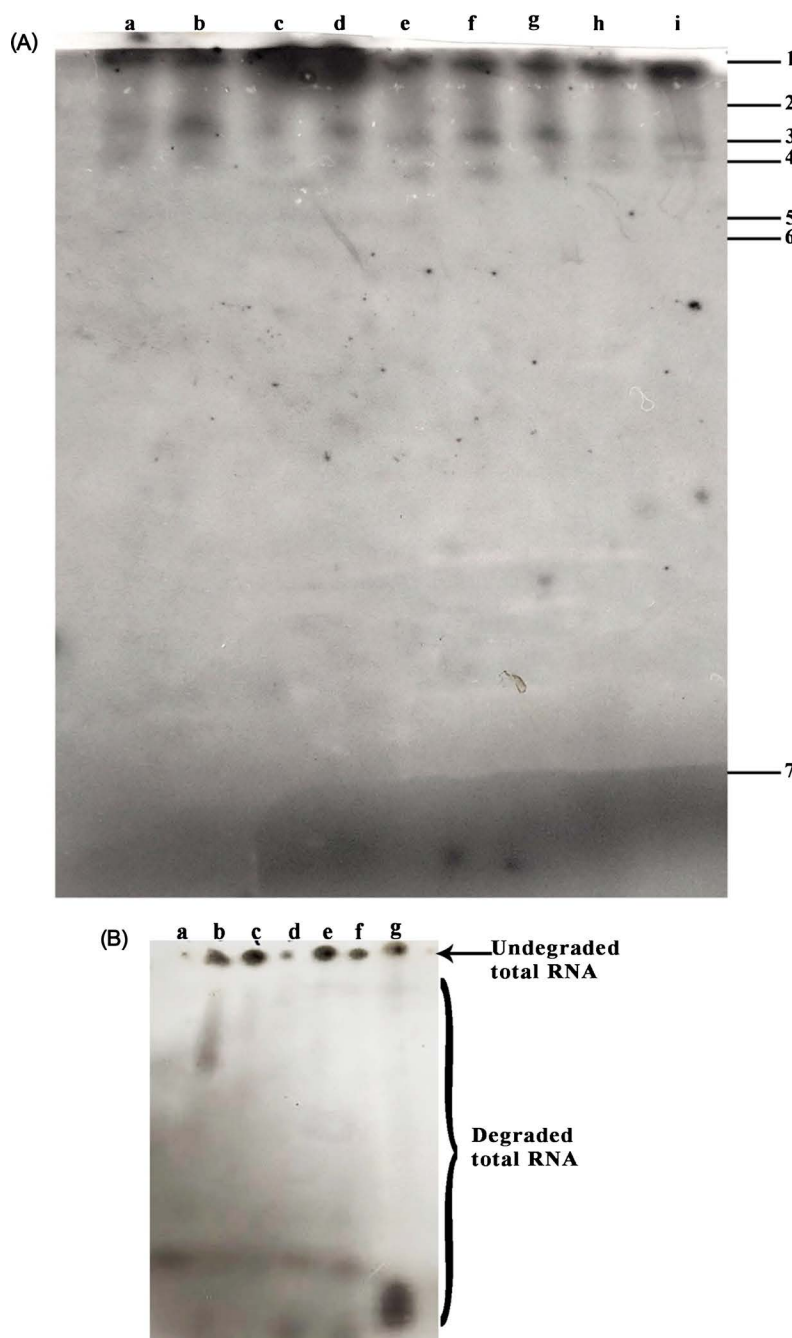
Figure 2. *In vitro* total RNA degradation products (polyacrylamide gel electrophoresis). GDH charge isomers in micro tubes (a) very acidic (pI 4.5); (b) acidic (pI 5.0); (c) mildly acidic (pI 6.0); (d) neutral (pI 7.0); (e) mildly alkaline (pI 8.0); (f) alkaline (pI 8.4); (g) very alkaline (pI 8.6) were purified by electrophoresis from P + K-treated peanut, and made to synthesize RNA enzyme. Peanut total RNA was 5' end-labeled with [γ^{32}P]-ATP. Labeled total RNA (20 μg) of KCl-treated peanut (A); and control peanut (B) was added to each micro tube containing RNA enzyme synthesized by each GDH charge isomer in 0.05 M Tris-HCl buffer solution pH 8.0. Micro-tubes (h), and (i) contained 20 μg of labelled total RNA in 0.05 M Tris-HCl buffer solution pH 8.0 but no GDH-synthesized RNA enzyme. Tubes (a), (b), (c), (d), (e), (f), (g), and (h) were thermo-cycled, but tube (i) was left on ice. After the thermo-cycling, 10 μL of each reaction was subjected to first-strand cDNA synthesis reaction using random hexamer primer and avian reverse transcriptase to generate RNA:DNA hybrids. After first-strand cDNA synthesis, half of the reaction solution was briefly electrophoresed through 7% urea polyacrylamide gel. The electrophoresed gel was vacuum-dried, and autoradiographed. In **Figure 2(A)**, band number 1 is undegraded total RNA; bands 2, 3, 4, 5, and 6 were mainly total RNA degradation products; and band 7 was mainly excess [^{32}P]-ATP. We ran out of labeled total RNA of control peanut for **Figure 2(B)** reactions (h) and (i).

the total RNA controls.

Much of the full length RNA: DNA hybrids of total RNA (substrate) were also stuck at the origin of the polyacrylamide gel, but all the GDH-synthesized isomeric RNA enzymes (very acid (pI 4.5 ± 0.2), acid (pI 5.0 ± 0.2), mildly acid (pI 6.0 ± 0.4), neutral (pI 7.0 ± 0.4), mildly alkaline (pI 8.0 ± 0.1), alkaline (pI 8.4 ± 0.3), and very alkaline (pI 8.6 ± 0.1) degraded the total RNA to different extents (**Figure 2(B)**). In addition to the fast migrating bands (excess ^{32}P-ATP, and low molecular weight oligonucleotides) near the bottom of the gel, there were smears of intermediate degradation products. Polyacrylamide gel electrophoresis (**Figure 2(B)**) of the RNA:DNA hybrids captured more of the intermediate degradation products than agarose gel electrophoresis of the total RNA (**Figure 1(B)**). The presence of intermediate degradation smears of total RNA was evidence for enzyme-substrate complexes in the degradation mechanism.

In the degradation of total RNA by GDH-synthesized RNA, the thermo-cycled control RNA was similar to the uncycled control RNA, and both were different from the degraded RNA patterns (**Figure 1**, **Figure 2**). Therefore, total RNA was not degraded by the thermo-cycling. Total RNA (substrate) was labeled at the 5' end. Therefore the degradation products that were visible were those at the 5' termini of total RNA moieties; those at the 3' termini being invincible to autoradiography. This differential detection simplified the visualization of the degradation products (**Figure 1**, **Figure 2**). Agarose gel and polyacrylamide gel electrophoresis showed that the preponderance of the degradation products co-electrophoresed with the excess labeled ATP, thus suggesting that most of the target RNA molecules were degraded to 5'-mononucleotides. The smear of degradation products captured on polyacrylamide gel (**Figure 2**) however showed that many of them were high molecular weight oligonucleotides. Labeled total RNA and its degradation fragments were converted to RNA:DNA hybrid (**Figure 2**) because RNA:DNA hybrids are known to migrate as compact bands under polyacrylamide gel electrophoresis [45] [46].

3.3. *In Vivo* Degradation of Total RNA by GDH-Synthesized RNA

Northern bands were obtained with phosphate translocator, flavonoid biosynthesis, granule-bound starch synthase, and 16S rRNA probes (**Table 2**) that were synthesized by GDH [37] [44] [47] [48] for all the experimental peanuts (**Figure 3**). Each probe was cDNA-labeled through PCR incorporation of [α-^{32}P]-dATP [5] [7] [23]. Each labeled cDNA probe produced at least a pair of Northern bands (**Figures 3(B)-(E)** typical of the structural characteristics of GDH-synthesized RNA to integrate and discriminate metabolic pathways [5] [21]. The mRNA (**Figure 3(B)**) encoding inorganic phosphate translocator (about 1700 bases long) [47] [48] was degraded *in vivo* by GDH-synthesized RNA enzyme in the control peanut, and in peanuts treated with P + S, P, N + S, N + P + K, and P + K stoichiometric mixes of mineral salt solutions (**Figure 3(B)**). The bands (about 1500 - 3000 bases long) in the lanes for N-, N + P + K + S-, K-, and S-treated peanuts were composite incompletely resolved multiplicity of bands

Table 2. cDNAs of some of the GDH-synthesized RNAs used as probes. The homologous section is bolded, the left flank is underlined, and the right flank is double underlined.

Inorganic phosphate translocator BAB16885.1

CTGGATNGCAGTTCGGCCGTTAAGGTTGGTCTTTGTTTAAGTTCCTTGTGGAAAGCCCTG
GGCTCATATTTTTGGGAACTCGCAGTGGA**TACTGGGCGACTAGAGTGTGGTAGAGGGT**
AGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGC
GAAGGCAGCTACCTGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAA
TTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCA
AGACTGAAACGCAAAGGAATTGACGGGTAAT

Flavonoid biosynthesis gene gb|EF165349.1|

CGGCATTGATAGCGATGAGTCCTGACCGACAACGGCATTGATAGCGATGAGTCCTGACC
GACAACGGCATTGATAGCGAT**GAGTCCTGACCGACAACGGCATTGATAGCGATGAGTC**
**CTGACCG**CAAGGCATTGATAGCGATGAGTCCTGA

Starch synthase IIa: gb|ACL98483.1|

GTTACGATTCGCCCTTATGAGTCCTGACCGAGAACCGCGTTGATGGGGATGAGTCCGGAC
CGCCAACGGCATTGATAACGATGAGTCTGGACGGAGCTTACTCTTTATAATGATGAGTCC
TGACCGACAACGGGTTTGATAGCTATGATTCCT**GACCGACTGCGGCATTGATAGCGATG**
AGTCTGGATGGGATATGCAGACTACCAGAACCTGATTGGCGACT

16 S ribosomal RNA gene EU982406.1; EU982414.1

TTAACGCGTTAGCTTCGATACTGCGTGCCAAATTGCACCCAACATCCAGTTCGCATCG**TT**
TAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCA
GTGTCAGTGTTGGTCCAGGTAGCTGCCTTCGCCATGGATGTTCCTCCTGATCTCTACG
CATTTCACTGCTACACCAGGAATTCCGCTACCCTCTACCACACTCTAGTCGCCCAGTA
TCCACTGCAGTTCCCAGGTTGAGCCCAGGGCTTTCACAACGGACTTAAACGACCACCT
ACGCACGCTTTACGCCCAGTAATTCCGAGTAACGCTTGCACCCTTCGTATTACCGCGG
CTGCTGA

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that included that of 1700 bases long for phosphate translocator mRNA. Phosphate translocator regulates the counter exchange of inorganic phosphate and triose phosphates between the chloroplast and cytoplasm, thereby controlling the flux of intermediates through the peanut citric acid cycle [21] [23] [39]. In the control peanut, and those treated with P + S, P, N + S, N + P + K, and P + K stoichiometric mixes of mineral salt solutions where the mRNA encoding phosphate translocator was degraded, the chloroplastic triose phosphates were converted to starch, and exported as maltose to the cytoplasm for utilization in cellulose biosynthesis biomass accumulation. Accordingly, all the peanuts where the phosphate translocator mRNA was degraded produced substantially more pod yields (9418 - 9822 kg per hectare) than those where the phosphate translocator mRNA was not degraded (6391 - 7266 kg per hectare) in agreement with earlier observations [49]. Peanut treated with N + P + K + S stoichiometric mineral salt mix produced a higher seed yield (12,513 per hectare) because the phosphate translocator mRNA was not degraded (**Figure 3(B)**) and granule-bound starch synthase mRNA was only partially degraded (**Figure 3(D)**) thus both biomass accumulation and glycolysis proceeded normally. This is the biochemistry-proven technology that buttresses and encourages the agricultural efforts of smallholder crop producers who cultivate their crops on healthy soil and harvest
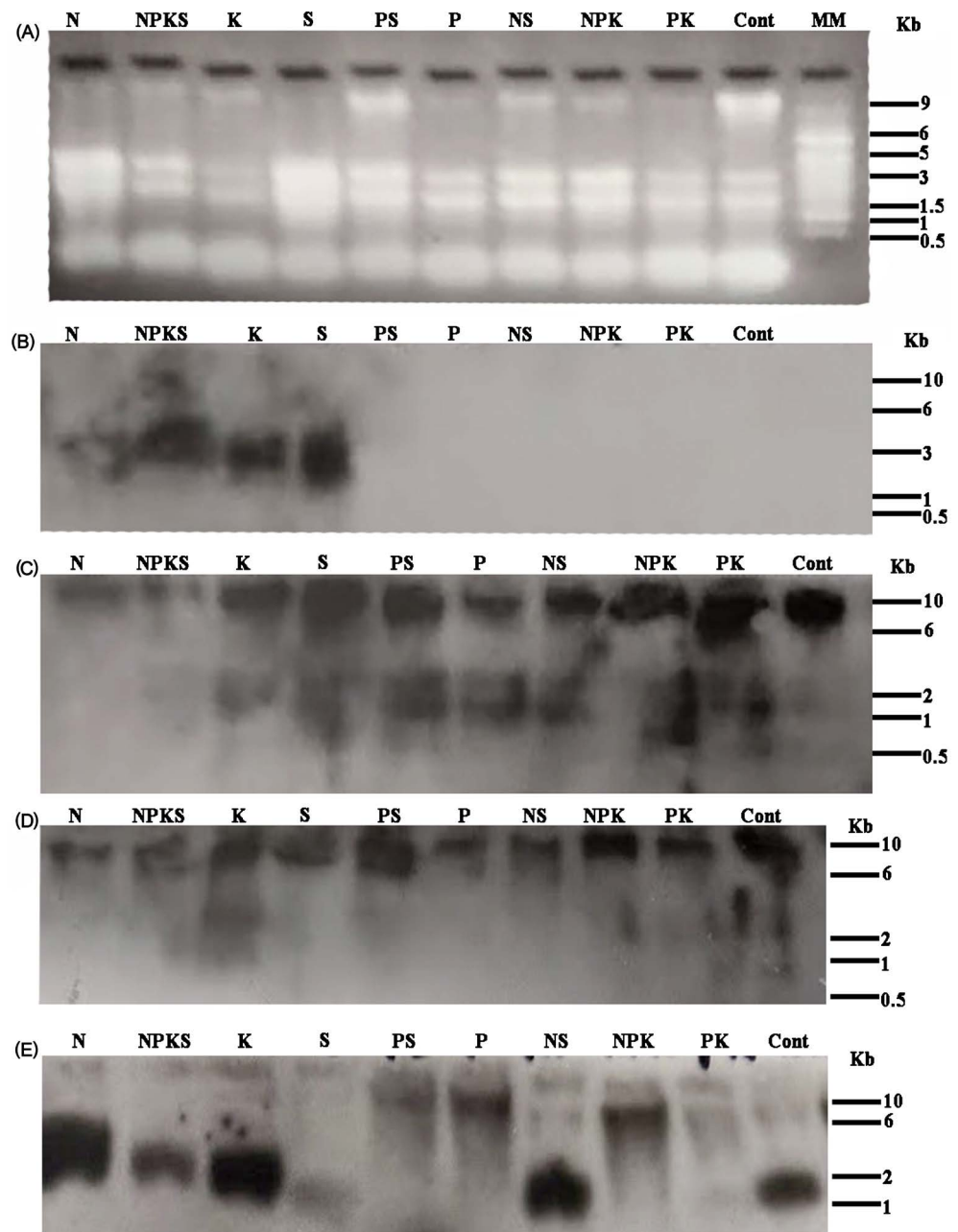
Figure 3. Peanut total RNAs and Northern blots demonstrating *in vivo* degradation of total RNAs. Total RNAs (A) extracted from the seeds of peanut fertilized with the indicated stoichiometric mixes of mineral salt solutions; electrophoresed through ethidium bromide-stained agarose gel, trans-blotted to nylon membranes, followed by membrane screening with ^{32}P-labeled cDNA of GDH-synthesized RNAs that were homologous to mRNAs encoding (B) inorganic phosphate translocator; (C) flavonoid biosynthesis enzyme; (D) granule-bound starch synthase; (E) 16 S rRNA. The membranes were washed with high stringency solutions and autoradiographed. MM means Ambion RNA millennium marker.

healthy crop yields despite the fact that they apply limited amounts of fertilizer to the crops. The integration and discrimination of triose phosphate transloca- tion and the starch synthase pathways (**Figure 3(B)** and **Figure 3(D)**) in the chloroplast by GDH-synthesized RNA enzyme leading to optimized biomass

yields [21] [39] is the corner stone of the agriculture innovation capacity of the more than 1 billion smallholder/limited resources crop farmers. The mRNAs encoding the flavonoid biosynthesis genes (about 9000 bases long) were not degraded (**Figure 3(C)**) in agreement with earlier observations [49] that horticultural peanuts treated with stoichiometric mixes of mineral salts have more flavor than commercially produced peanuts. The 16S rRNA bands (1800 bases long) were differentially degraded (**Figure 3(E)**) in the peanuts treated with S, P + S, P, and P + K. stoichiometric mineral salt mixes. Therefore, what was observed in the *in vitro* degradation of total RNA (**Figure 1**, **Figure 2**) agreed with the *in vivo* degradation of mRNAs and rRNA (**Figures 3(B)-(E)**) in that in both cases total RNA was degraded.

3.4. Mechanism of Total RNA Degradation by GDH Isoenzymes

All the GDH-synthesized Northern probes (**Table 2**) recognized their target mRNAs or rRNA not on the basis of the genetic code-based structures of total RNA but on homologous sequence alignments. When the non-homologous RNA sequences that flanked the homologous sequences were clipped out (**Table 2** and **Table 3**), and the complementary oligonucleotides were used as Northern probes, there was no recognition of the total RNA targets and there were no Northern bands. Therefore, the total RNA degradation function of GDH-synthesized RNA is independent of the genetic code.

The chemically synthesized probes (**Table 3**) that corresponded to the GDH-synthesized probes did not give any Northern bands thereby revealing the chemical mechanism of the degradation of total RNA. The role of the flanking sequences is therefore to direct, guide, and align the homologous sequence in the GDH-synthesized RNA to the target site in the homologous total RNA sequence.

The G + C contents [7] of GDH-synthesized RNA (non-genetic code-based RNA) are different from those of total RNA (genetic code-based RNA). On the

Table 3. Some chemically synthesized oligonucleotide probes.

Oligo probe for mRNA encoding inorganic phosphate translocator:

CTCCCCACGCTTTCGTGCCTCAGTGTCAGTGTTGGTCCAGGTAGCTGCCTCGCCATGGAT GTTCCTCCTGATCTCTACGCATTTCACTGCTACACCAGGAATTCCGCTACCCTCTACCAAC CACTCTAGTCGCCAGTAT

Oligo probe for mRNA encoding flavonoid biosynthesis gene:

CGGTCGGACTCATGTCGGTCAGGACTCATGTCGGTCAGGACTCATGTCGGTCAGGACTCA

Oligo probe for mRNA encoding starch synthase IIa:

ACTCATCGCTATCAATGCCGCAGTCGGTCA

Oligo probe for 16 S rRNA

CGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAG GTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACT GGGCGACTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAG AGATCAGGAGGAACATCCATGGCGAAGGCAGCTACTGGACCAACACTGACACTGAGGCA CGAAAGCGTGGGGAGCA AACAGGATTAGATACCCTGGTAGTCCACGCCCTAAA

basis of their different G + C contents, the electro-magnetic properties of total RNA are different from those of GDH-synthesized RNA. The GDH-synthesized RNA probe that is homologous to the mRNA encoding phosphate translocator is repeated two times in the range from nucleotide residue 151 - 347 of the mRNA. The GDH-synthesized probe that is homologous to the mRNA encoding starch synthase is repeated four times in the range from nucleotide residue 110 - 651 of the mRNA. The GDH-synthesized probe that is homologous to 16S rRNA matches once with 16S rRNA in the nucleotide range from residue 418 - 696. The GDH-synthesized RNA probe that is homologous to the mRNA encoding flavonoid biosynthesis enzymes is repeated six times in the range from nucleotide residue 18 - 157 of the mRNA. The matches and multiple repeats of the GDH-synthesized RNA within the zone of homology of the genetic code-based RNA probably facilitate homologous alignment reaction between the two types of RNA, and so make the machinery and chemistry of degradation completely different from the double-stranded RNA-mediated co-suppression post-transcriptional gene silencing that also embodies the participation of protein enzyme complexes [15] [50].

When the GDH-synthesized RNA aligns to the homologous target genetic code-based RNA (Figure 4), the resulting electro-magnetic collision (electrostatic repulsion) between them leads to the degradation of the homologous genetic code-based RNA, which is the lesser stable of the two kinds of RNA (Figure 4). Removal of the structural constraint imposed by genetic code transformed RNA to a fully-fledged RNA enzyme that is independent of genetic code for its biological function. Genetic code-based nucleic acids are thermally less stable [7] than non-genetic code-based nucleic acids. GDH-synthesized RNA enzyme is above the genetic code.

Failure of the chemically synthesized oligonucleotide to hybridize to the target total RNA sequence is evidence that base pairing hydrogen bonding is not the chemical mechanism of alignment and degradation of total RNA by GDH-synthesized
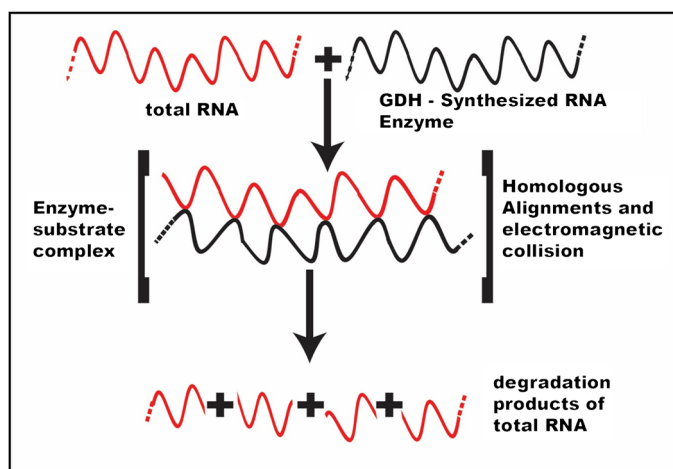
Figure 4. Schematic description of the chemical degradation of total RNA by GDH-synthesized RNA enzyme.

RNA. The alignment of GDH-synthesized RNA to the target genetic code-based RNA is by homologous sequence interaction, involving non-canonical base-pair formation between the two kinds of RNA. Non-Watson Crick base pairs of the types AA, UU, GG, CC, AU, GU etc that are involved in homologous sequence alignment also include van der Waals, electrostatic, and solvation terms that are known to stabilize RNA structural motifs and their helix arrangements [8] [9] [10] [11] [12]. However, the non-canonical base-pair formation in homologous RNA alignment is weaker than Watson Crick complementary hydrogen bonds, thus explaining the choice of low temperatures that were applied in the *in vitro* degradation of total RNA by GDH-synthesized RNA. Therefore, degradation of total RNA by non-genetic code-based RNA (GDH-synthesized RNA) confirms the nucleic acid chemistry [51]. Furthermore, double-stranded RNAs (miRNA, siRNA, shRNA etc), RISH and RITS protein complexes that dominate RNA interference mechanisms [16] [17] were not involved in the GDH-synthesized RNA machinery (Figure 4). Homology-dependent gene silencing has been described [52] but it focused on transgenes without explaining the chemical mechanism of silencing. The fact that total RNA degradation fragments formed RNA:DNA hybrids (Figure 2) suggested that the degradation mechanism was neither depurination nor depyrimidination of total RNA.

### 3.5. Biochemistry-Proven Technology That Supports Smallholder Farmers

The differential degradation of mRNAs, tRNAs, and rRNAs (Figures 1-4) shed light on the biochemical mechanism by which crops survive when they are cultivated by limited resource farmers. Sanchez-Calderon [53], and Pang *et al.* [54] observed that deficiencies in soil mineral nutrients increased the accumulation of leaf and seed biomass in agreement with Osuji *et al.*, [1] [22] [41] who had reported the GDH enhancement of biomass yield in maize and soybean treated with stoichiometric mixes of mineral salt solutions. Smallholder, limited resource, and indigenous farmers lack the financial power to purchase enough of expensive fertilizers, pesticides, and agricultural machinery in support of their crop production efforts, but some of the time they still harvest lots of food and feed stuff to nourish and to generate income for their families. Most of their farm lands are degraded and infertile. Many studies in plant physiology had focused on the transcriptional and biomass changes associated with mineral nutrient imbalance [24] [25] [55] [56] [57]; but the authors assumed and speculated that the transcriptional changes optimized translational processes for the increased biomass yield. The biochemical mechanisms that created the differential abundance of the mRNAs, proteins, and enzyme activities were not discussed. There is need to enhance the science-based agriculture innovation capacity of smallholder indigenous farmers.

Therefore, the mechanism is that when plants are subjected to soil mineral nutrient deficiencies by limited resource farmer, the plant's GDH-synthesized RNA enzymes quickly degrade superfluous mRNA, tRNAs, and rRNAs (Figures

1-4) thereby minimizing the wastage of metabolic energy in the synthesis of unnecessary proteins, but optimizing the synthesis of needed amino acids, proteins and enzymes that assure development, nutritious biomass phytochemical accumulation (for plant protection), and survival of the crop. These are the biochemically proven reactions that support the agriculture innovation capacity of smallholder farmers. The mechanisms also underlie the biotechnology by which indigenous limited resource farmers, ethno-botanists, traditional herbalists, and practitioners of alternative medicines in Africa and Asia generate metabolic variants of ancient medicinal plants and spices that are enriched in specific varieties of pharmacologically active phytochemicals that support human health, without genetic engineering or plant breeding alteration of the plants. Amino acid biosynthesis [5] and translation are the most energy consuming processes in the cell [58]. Biomass metabolism requires protein synthesis and production of ribosomal subunits, tRNAs, and translational factors that are rigidly controlled at transcriptional and post-transcriptional levels [59]. The swift degradation of superfluous total RNA by GDH-synthesized RNA enzyme (**Figures 1-4**) in response to biotic and abiotic stressors, growth and differentiation cues of the crop results in much more immediate cellular adjustment through direct regulation of protein accumulation. The other mechanisms [13]-[20] that also control total RNA activities are housekeeping processes that are not sensitive to the crop's metabolic environment. When we subjected peanut to K + N stoichiometric mineral salt treatment (mineral nutrient deficiency/imbalance), the peanut produced a mega pod yield of 12,780 kg per hectare [49] compared with the USA peanut producer yield of 3184 - 5936 kg per hectare [60] achieved with traditional time consuming, and laborious crop rotation and application of inactive fertilizers. This extraordinary > 200% increase in crop yield is the R&D application of GDH-synthesized RNA enzyme crop yield doubling biotechnology for the enhancement/optimization of food crop yields [23]. Peanut production is a worldwide gold mine in agriculture because peanut oil earns about U$1470 per ton in the world market, more than double of soybean oil [61]. GDH-synthesized RNA enzyme crop yield-doubling biotechnology has the promise to minimize not only hunger but also the cost of agricultural production by limited resource, smallholder farmers, and city community gardeners.

3.6. Purification of GDH Isoenzymes

As the enzyme that synthesizes non-genetic code-based RNA, the GDH isoenzymes are naturally attached to some RNA. Therefore, it is required to treat tissues with RNase A during GDH isoenzyme purification in order to hydrolyze all the RNA. All the methods that purified GDH by chromatography did not remove the bounded RNA from the GDH isoenzymes [34] [35] [36] [62] [63]. The differences in the molecular distributions of GDH isoenzymes on native polyacrylamide gel landscape when RNAs are bounded to them and when the RNAs have been removed are astounding because more acidic isoenzymes are released after RNase treatment of GDH (**Figure 5**). The isoenzymes became up to ten

Rotofor IEF Chambers
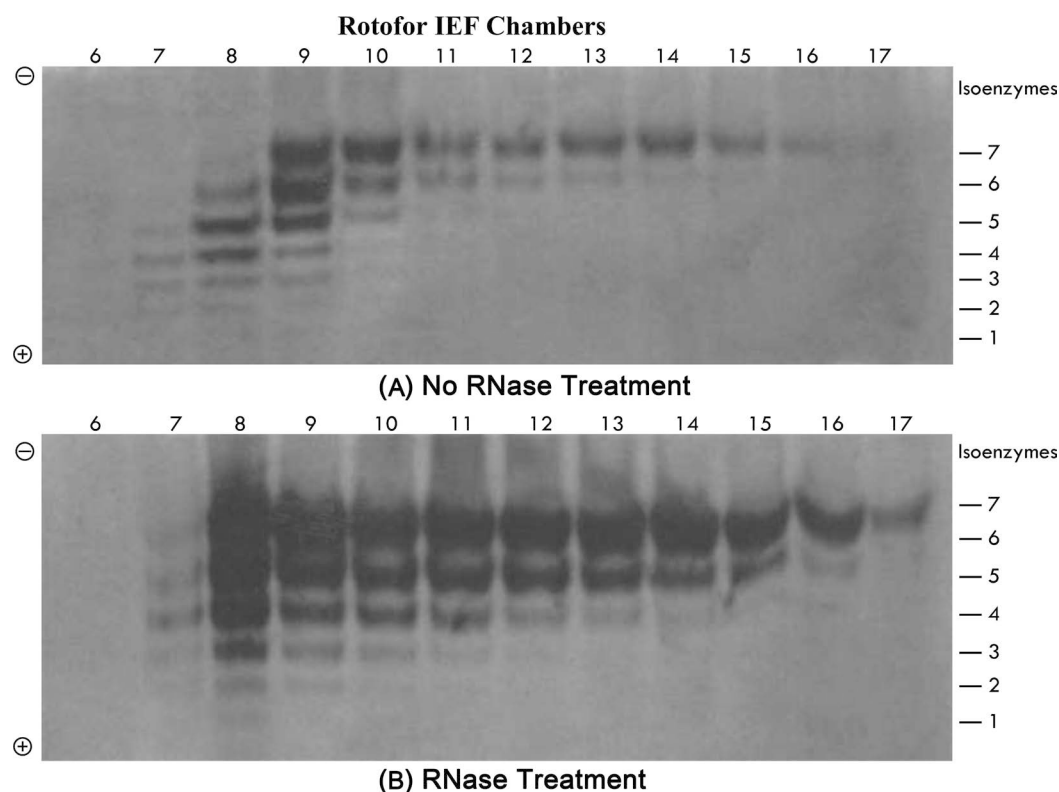
Figure 5. Glutamate dehydrogenase (GDH) isoenzyme profiles before and after RNase treatment. GDH isoenzyme was isolated from equal weights of peanut seeds harvested from N-Carboxymethyl chitosan-treated raised beds; using Tris-HCl buffer cocktails without RNase A (5A), and containing RNase A (5B). In each case, an equal volume of the GDH was subjected to Rotofor isoelectric focusing (IEF) to its vertical bands of charge-isomers. The pI values of the Rotofor fractions were determined before urea and ampholyte were removed by dialysis. Then, equal volumes of dialyzed Rotofor fractions 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, and 17 were fractionated to the horizontal bands of isoenzymes (1), (2), (3), (4), (5), (6), and (7) by native polyacrylamide gel electrophoresis. The GDH isoenzyme profile was visualized by activity staining of the electrophoresed polyacrylamide gel with tetrazolium bromide reagent. **Figure 5(A)** is the natural aminating profile of GDH isoenzymes bounded to RNA; **Figure 5(B)** is the aminating profile of the liberated GDH isoenzyme.

folds more aminating after they were liberated from the bounded RNA judging from the digitalized intensities of the isoenzyme bands (**Figure 5**).

GDH is multi-isoenzymic, therefore it is required in enzymology to purify the intact isoenzymes rather than the subunit polypeptides. The electrophoretic purification by free solution isoelectric focusing concentrates the isoenzymes into a few chambers of the Bio-Rad's Rotofor cell; the native polyacrylamide gel electrophoresis of the Rotofor fractions removes all RNAs and other proteins because of the high molecular weight of GDH isoenzymes [1] [22] [41]; gentle cryoelectrophoresis of the slab of native polyacrylamide gel elutes the isoenzymes thus assuring homogenous isoenzyme preparations that are not dissociated and fragmented to subunit polypeptides. This multi-dimensional electrophoretic procedure has been applied to purify not only *Arachis hypogeae* GDH isoenzymes but also human cells (Professor Aubrey Thompson Laboratory), and *Zea mays* [64] [65] [66] GDH isoenzymes. In all cases, the homogenous GDH

isoenzymes were applied to prepare polyclonal antibodies which neatly detected GDH isoenzymes and subunit polypeptides on Western blots [2].

The GDH isoenzymes purified from control untreated peanut, KCl-treated, and P + K-treated peanut were unique and different from each other, but they displayed the standard binomial distribution patterns of isoenzymes on native polyacrylamide gel (**Figure 5**, **Figure 6**). Similarly, the RNAs synthesized by the charge isomers of the GDHs of control peanut, KCl-treated peanut, and P + K-treated peanut (**Figure 7**) were typical of those synthesized by peanut GDH [23]. The total RNA extracted from all the experimental peanuts (**Figure 3(A)**) were free from RNase contamination because the rRNA bands were vividly present in consistent ratios.

4. Discussion

GDH-synthesized RNA enzyme: Above and beyond the genetic code. Degradation of the total RNA of KCl-treated peanut by the GDH-synthesized RNA of control peanut showed that KCl-treated, P + K-treated, and control peanuts were different metabolic variants of *Arachis hypogeae*, GDH-synthesized RNA being a simple analytical tool for demonstrating phenotypic difference. The differential activity towards tRNAs, 5S rRNA, 16S rRNA, and 26S rRNA could also be applied to depopulate rRNAs and tRNAs in the R&D analysis of genomic
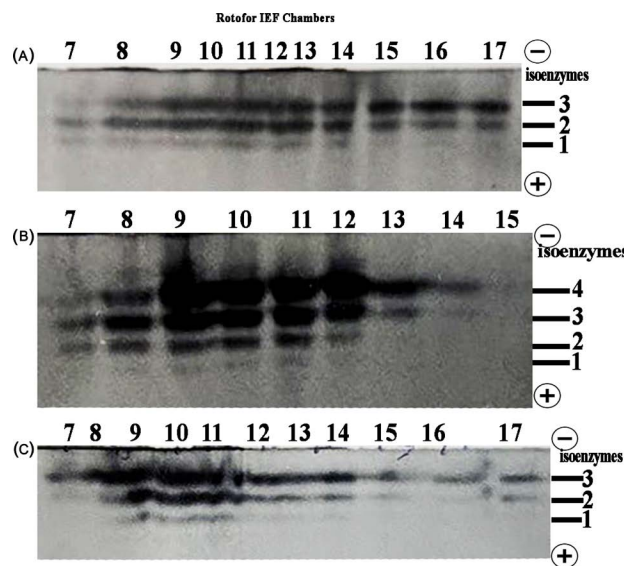
Figure 6. Stoichiometric mineral salt-induced isomerization of peanut GDH. GDH isoenzyme was isolated from equal weights of peanut seeds harvested from (A) P + K-treated; (B) Control-untreated; (C) KCl-treated peanuts; using Tris-HCl buffer cocktails with RNase A. In each case, an equal volume of the GDH was subjected to Rotofor isoelectric focusing (IEF) to its vertical bands of charge-isomers. The pI values of the Rotofor fractions were determined before urea and ampholyte were removed by dialysis. Then, equal volumes of dialyzed Rotofor fractions 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, and 17 were fractionated by native polyacrylamide gel electrophoresis to the horizontal bands of isoenzymes (1), (2), (3), (4). The GDH isoenzyme profile was visualized by activity staining of the electrophoresed polyacrylamide gel using tetrazolium bromide reagent.
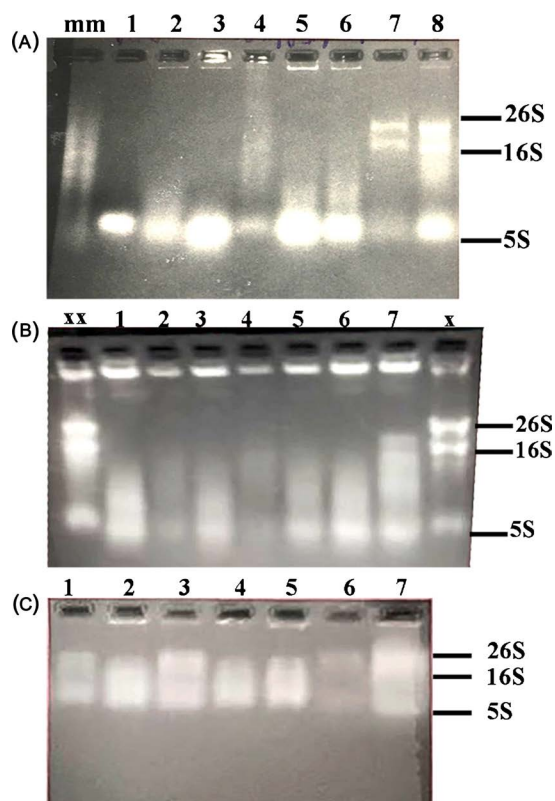
Figure 7. RNA enzymes synthesized by GDH charge isomers. The GDH charge isomers 1) very acidic (pI 4.5); 2) acidic (pI 5.0); 3) mildly acidic (pI 6.0); 4) neutral (pI 7.0); 5) mildly alkaline (pI 8.0); 6) alkaline (pI 8.4); 7) very alkaline (pI 8.6) eluted from the slab of native 7.5% polyacrylamide gel were used for RNA synthesis in cocktails containing the four NTP mixes, DNase 1, RNase inhibitor and modulators. The RNA products were electrophoresed through ethidium bromide-stained 2% agarose gel. The RNA enzymes synthesized by the GDH charge isomers of P + K-, Control-, and KCl-treated peanuts are shown in (A) - (C) respectively. X is 10 μg and XX is 20 μg of the total RNA of control peanut; mm is Ambion RNA millennium marker.

RNA. Currently, only Roche KAPA RiboErase kit presents a molecular biology method for the removal of rRNAs from some mammalian total RNA preparation. RNA synthesized by the different charge isomers (very acid, acid, mildly acid, neutral, mildly alkaline, alkaline, very alkaline) of GDH were differentially active in the degradation of total RNA (Figure 1, Figure 2) because the GDH hexameric subunit compositions are different [47] [48], and accordingly the primary structures of the RNA enzymes they synthesized were different [7]. The degradation of total RNA (Figure 1, Figure 2) demonstrated that GDH-synthesized RNA enzyme, similar to GDH is a group of isomeric enzymes with different specificities towards total RNA sequences. The differential activity of the isomeric RNA enzymes towards total RNA constituents (Figures 1-4), is the biochemical mechanism by which GDH functions *in vivo* to integrate and discriminate environmental redox signals [4]. Rotofor isoelectric fractionation of GDH to isoenzymes was very important for the visual demonstration of the differential degradative activities of the isomeric RNA enzymes towards total RNA.

For the degradation of total RNA by GDH-synthesized RNA, total RNA (substrate) was labeled at the 5' end. Therefore, the degradation products that were visible were those at the 5' termini of total RNA moieties; those at the 3' termini being invincible to autoradiography. Native polyacrylamide gel electrophoresis showed that the preponderance of the degradation products co-electrophoresed with the excess labeled ATP, thus suggesting that most of the target RNA molecules were degraded to 5'-mononucleotides. The smear of degradation products captured on polyacrylamide gel (**Figure 2**) suggested that the GDH-synthesized RNA possessed endonuclease activity that progressed towards the 5' end of the substrate RNA molecule. This specificity could be exploited to further illuminate mRNA, rRNA, tRNA sequence organization. It is known that mRNA undergoes decay by two pathways [14]. Instantaneous degradation of superfluous total RNA by GDH-synthesized RNA (**Figures 1-7**) offers another R&D approach for investigating RNA structural organization in relation to the mechanisms of total RNA decay. Agarose gel electrophoresis (**Figure 1**) showed that the low molecular weight (26S rRNA, 16S rRNA, 5S rRNA, and tRNA) moieties of total RNA were the easiest targets of degradation by GDH-synthesized RNA enzyme. Ribosomal RNAs and tRNAs are among the major genetic code-based RNAs that regulate translation processes [29] [67]. GDH isomerization regulates plant growth and differentiation [1] [2] [41]. Therefore, by differential degradation of mRNAs, and rRNAs, GDH-synthesized RNA enzyme holds a firm grip on translation (protein biosynthesis).

In vitro degradation of total RNA by GDH-synthesized RNA enzyme (**Figure 1**, **Figure 2**) is a phenomenal prototype demonstration of R&D methods for cleansing sick total RNAs from cells, tissues, organs, whole organism because the enzyme is above and beyond the genetic code.

Acknowledgements

Conflicts of Interest

The authors declare no conflict of financial interest on the project.

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