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INHIBITION OF STABLE RNA SYNTHESIS AND PRODUCTION OF A NOVEL RNA IN HEAT-STRESSED PLANTS

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ABSTRACT. In tobacco and cowpea leaves incubated at elevated temperatures, incorporation of ^{32}P or ^{3}H-uridine into cytoplasmic rRNA and 5S RNA was inhibited while that into tRNA continued and additionally a novel RNA species of approximately 0.49 x 10^6 daltons was produced. This RNA was single stranded with a base ratio of 33/29/23/14 (A/U/C/U) and appears not to possess a poly (A) sequence. It hybridized to total plant DNA with kinetics suggesting a unique sequence origin and synthesis was suppressed by actinomycin D. Hybridization was competed by RNA from plants maintained only at 25°C, suggesting that low levels of this RNA occur in plants at normal temperatures even though it is not labeled detectably.

INTRODUCTION. The ability to specifically manipulate one or a few genes could greatly simplify attempts to understand regulation of gene expression in plants. Such systems have been developed in animal systems utilizing elevated temperatures. Upon shift of Drosophila tissues to 37°C, the pattern of protein synthesis is markedly altered (1,2,3). These changes are due to inhibition of transcription of most pre-existing mRNA along with the production of approximately 6 new species of mRNA (4), with one (9 X 10^5 daltons) found predominantly in polysomes producing a 70,000 dalton heat-stress protein (5). These changes coincide with new visible puffs in the chromosome to which the new mRNAs specifically hybridize in situ (4,5). This system is greatly facilitating the understanding of the relationship of chromosome structure to gene expression (6,7).

In this paper, we examine RNA synthesis in tobacco and cowpea leaves at elevated temperatures, and report that syntheses of the different stable

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RNAs react differently to heat stress. Cytoplasmic rRNA and 5S RNA syntheses were inhibited while transfer RNA synthesis continued. Additionally, a new RNA that is produced at the elevated temperatures is described.

MATERIALS AND METHODS. Labeling procedure: Tobacco (Nicotiana tabacum L. var. Xanthi) and cowpea (Vigna unguiculata (L.) Walp. var. California Blackeye) plants were soil-grown in a glasshouse at 20-25°C. Leaves were detached and vacuum infiltrated in 60 μCi/ml $H_3{}^{32}PO_4$ in 0.1 mM KPO_4 buffer, pH 7.0, or 67 μCi/ml [^{3}H] uridine in distilled water after which they were incubated at the designated temperature in full light. Labeling was terminated by freezing the tissue at - 20°C.
Nucleic acid extraction: Tissue frozen in liquid nitrogen was powdered with a mortar and pestle and nucleic acids were extracted with phenol and analyzed as described previously (8). DNA was purified by agarose gel (Sepharase 2B, Pharmaacia) chromotography as described by Petrovic et al (9).
Hybridization conditions: DNA extracted as described above from tobacco plants maintained at 25°C was sheared as described by Kang and Temin (10) followed by incubation at 100° for 10 min and cooling on ice. Trace amounts of ^{3}H-labeled 0.49 X 10^6 dalton RNA was incubated with excess DNA in 0.12 M KPO_4 buffer, pH 6.8, at 60°C. Reactions were terminated by freezing at -80°C. Samples then were thawed, 20 X SSC (0.15M NaCl, 0.015M sodium citrate, pH7.0) was added to a final concentration of 3 X SSC. Samples then were incubated with or without RNase (10 μg/ml RNase A plus 10 units/ml RNase T_1) at 37°C for 1 hr. The samples then were spotted onto 3mm filter paper discs (Whatman) and washed (11) and counted (12) as described.
Labeling and extraction of proteins: Leaves were detached and vacuum infiltrated in 50 μCi/ml ^{3}H-leucine followed by incubation in petri dishes at 25°C or 40°C for 4 hrs as described above and freezing at -20°C. Protein was extracted (13) and analyzed by SDS slab gel electrophoresis and fluorography (14).

RESULTS. Synthesis of stable RNAs at elevated temperatures: Young tobacco leaves were incubated in the presence of ^{32}P for 2 hr at different temperatures. At 25°C, ^{32}P was incorporated predominantly into cytoplasmic rRNA (1.3 and 0.7 X 10^6 daltons) and tRNA (Fig. 1A). When tissue growing at 25°C was shifted to 37°C and labeled with ^{32}P, incorporation into cytoplasmic rRNA was slightly reduced, but incorporation into tRNA was not (Fig. 1B). In addition, a new prominent peak of incorporation that migrated more rapidly than rRNA was found in tissue labeled at 37°C. When leaves growing at 25°C were labeled at 40°C, incorporation into tRNA increased, but incorporation into cytoplasmic rRNA was drastically reduced (Fig. 1C). Additionally, the incorporation into the rapidly migrating peak increased. When the leaves were pre-incubated at 40°C (up to 12 hr) prior to being labeled at 40°C, the rates

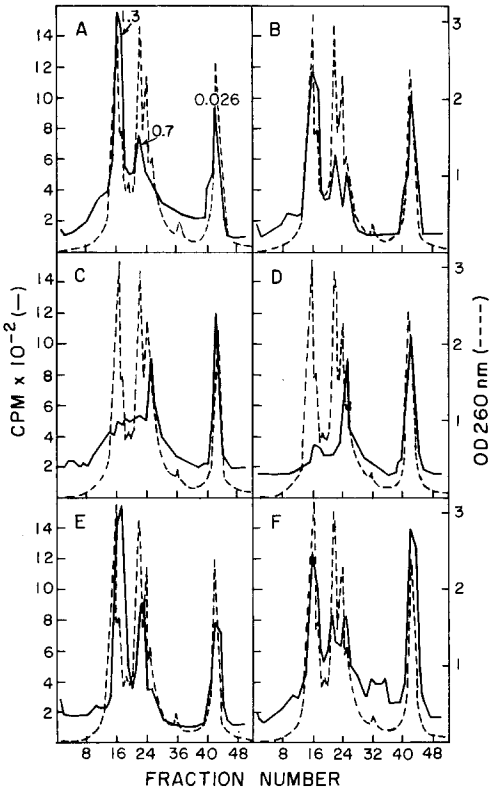

Fig. 1. Effect of temperature upon incorporation of ^{32}P into stable RNAs. Samples were fractionated in 2.5% polyacrylamide gels for 2 hr at 7 mA/gel. Tobacco leaves were grown at 20-25°C and were incubated with ^{32}P for 2 hr at 25°C (A), 37°C (B), 40 C (C), pre-incubated for 2 h at 40°C and incubated 2 hr at 40°C with ^{32}P (D), pre-incubated 12 hr at 40°C and incubated 2 hr at 25°C with ^{32}P (E), and incubated with ^{32}P for 2 hr at 25°C and 2 hr at 40°C (F). Numbers over peaks refer to molecular weight in millions.

of incorporation into tRNA and the new rapidly migrating peak remained constant, but the amount of incorporation into rRNA was reduced further (Fig. 1D). However, when plants were incubated at 40°C for up to 24 hr before being returned to 25°C and incubated with ^{32}P, the incorporation into rRNA and tRNA was the same as in plants incubated only at 25 C (Fig. 1E). The stablity of newly synthesized rRNA was examined by analyzing RNA from tissue labeled 2 hr at 25°C and shifted to 40°C for 2 additional hr (Fig. 1F). The amounts of incorporation into cytoplasmic rRNA were similar to that from leaves labeled only 2 hr at 25°C. Incorporation into tRNA was greater, approximately the additive incorporations of 2 hr at 25°C (Fig. 1A) and 2 hr at 40°C (Fig. 1C).

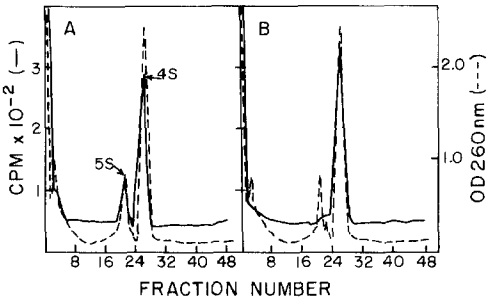

Fig. 2. Effect of temperature upon incorporation of ^{32}P into 4S and 5S RNA. Samples were fractionated in 7% polyacrylamide gels for 3 hr at 7 mA/gel. Tobacco leaves were grown at 20-25°C and were incubated with ^{32}P for 2 hr at 25°C (A) or 40 C°(B).

The newly synthesized rRNA did not break down appreciably at 40°C. Similar experiments with cowpea leaves gave almost identical results. Incorporation of ^{3}H-uridine into the RNAs of both tobacco and cowpea leaves was identical to the ^{32}P profiles.

At 40°C, incorporation into 5S RNA was reduced similarly to the inhibition of rRNA synthesis (Fig. 2). Transfer RNA was labeled rapidly at 40°C with both ^{32}P and [^{3}H]uridine. The base ratio of ^{32}P tRNA labeled at 25°C was 19/24/26/29 (A/U/G/C) which is near the expected ratio. However, ^{32}P tRNA labeled at 40°C had an altered ratio, 16/26/21/37 (A/U/G/C).

Properties of the novel RNA: The molecule responsible for the novel peak of radioisotope incorporation was extracted from polyacrylamide gel fractions and analyzed for nuclease susceptibility. This material was resistant to DNase (50 g/ml, 10mm $MgCl_2$, 1 hr at 37°C) but was digested totally by RNase (5 μg/ml RNase A plus 0.5 μg/ml RNase T_1, 30 min at 37C) in both 0.02M NACl and 0.2M NACl suggesting that it was single-stranded RNA. This is supported by its precipitation in 2.0 N LiCl. Also, incorporation of 32P into this novel RNA was prevented by levels of actinomycin D (30-50 μg/ml) that suppress incorporation into rRNA in these leaves but in which viral RNA synthesis continues (8). The estimated molecular weight of the novel RNA based upon the migration rates rRNA and the 4 components of cowpea chlorotic mottle virus RNA during electrophoresis in 2.5, 3.0, and 3.5% plyacrylamide gels was 0.49 X 10^6 daltons. The base ratio was 33/29/22/14 (A/U/G/C).

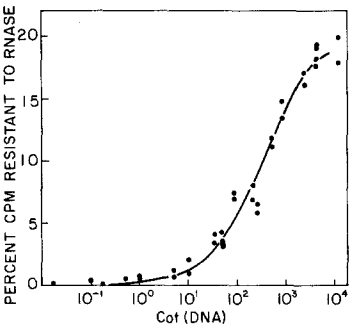

Fig. 3. Hybridization of trace amount of 0.49 X 10^6 dalton RNA to tobacco DNA as described in Materials and Methods.

The 0.49 X 10^6 dalton RNA was analyzed for the presence of poly (A) sequences by several procedures. It was retained by an oligo (dT) column by the procedure of Aviv and Leder (15). In one experiment 1013 cpm were retained while 127 cpm were excluded. However, it did not hybridize detectably to poly (U). Upon complete digestion of ^{32}P-labeled 0.49 X 10^6 dalton RNA by RNase A plus RNase T_1 and analysis by electrophoresis in 10% polyacrylamide gels as described by Key and Silton (16), there was no evidence of a poly (A) peak. Only the mononucleotide peak was observed. These data suggest that this RNA does not contain poly (A) sequences and that the oligo (dT) binding was due to the high (A) content.

The kinetics of hybridization of trace amounts of the 0.49 X 10^6 dalton RNA to excess tobacco DNA is shown in Figure 3. The novel RNA appeared to be half-hybridized between Cot values of 0.3-1.0 X 10^3 or greater, suggesting that this RNA results from the single-copy class of DNA sequences.

To determine whether RNA sequences common to the 0.49 X 10^6 dalton RNA accumulated in plants maintained only at 25°C, excess amounts of different fractions of RNA were incubated with trace amount of ^{3}H-labeled 0.49 X 10^6 dalton RNA in hybridization reactions to DNA (Table 1). Total 2N LiCl soluble RNA did not compete the hybridization reaction whereas total 2 N LiCl insoluble RNA did inhibit the reaction. When RNA was extracted from gels and used in the competition reaction, only the RNA that migrated in the same region as the

TABLE 1: Hybridization of tract amount of ^{3}H-0.49 X 10^{6} dalton RNA to DNA in the presence of excess unlabeled RNA.

Input 0.49 X 10^{6} dalton RNA, cpm	Input Competitor RNA, μg	RNase Resistant cpm after hybridization minus background
2000	0	472
2000	2 (LS RNA)[a]	481
2000	20 (LS RNA)	358
2000	200(LS RNA)	373
1250	0	398
1250	11 (LP RNA)[b]	119
1250	88(LP RNA)	47
1250	219(LP RNA)	35
1700	0	242
1700	4 (>0.56 RNA)[b]	283
1700	40 (>0.56 RNA)	359
1700	400(>0.56 RNA)	383
1700	3 (0.56-0.47 RNA)	230
1700	27 (0.56-0.47 RNA)	227
1700	270(0.45-0.47 RNA)	131
1700	3 (<0.47 RNA)	378
1700	30 (<0.47 RNA	250
1700	300(<0.47 RNA)	217

[a] Input DNA was 625 g.

[b] LS = total 2N LiCl soluble RNA; LP = total 2N LiCl precipitable RNA; 70.56, 0.56-0.47, and <0.47 = LP RNA extracted from acrylamide gels after electrophoresis, numbers are X 10^{6} dalton.

0.49 X 10^{6} dalton RNA interfered with the hybridization reaction. This suggests that the 0.49 X 10^{6} dalton RNA is synthesized and accumulates to lower levels in plants at normal temperatures.

In vivo protein synthesis at elevated temperatures: Figure 4 shows the profile of ^{3}H-leucine incorporation into total protein at 25°C compared to

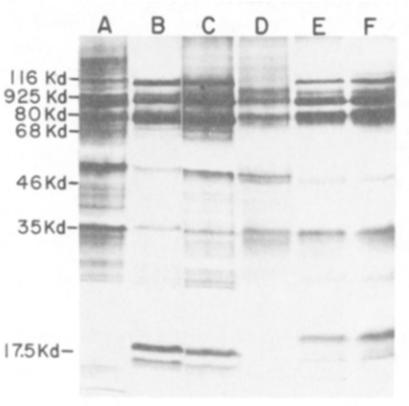

Fig. 4. Fluorogram of incorporation of ^{3}H-lencine into total protein of tobacco (A,B,C) and cowpea (D,E,F) labeled 4 hr at 25°C (A,D), 40°C (B,E), and pre-incubated at 40°C for 24 hr before labeling 4 hr at 40°C (C,F).

40°C. Synthesis of proteins of approximate molecular weights of 100kd, 90kd, 80kd, 18kd (cowpea), and 17kd (tobacco) was markedly stimulated while synthesis of other proteins was increased to a less extent. This was paralleled by the reduction of synthesis of many proteins normally produced at 25°C. However, neither the sizes nor the number of proteins stimulated by heat stress were comparable to an expected protein using the 0.49 X 10^{6} dalton RNA as messenger.

DISCUSSION. When tobacco or cowpea leaves that were grown at 20-25°C were shifted to 40°C and incubated with ^{32}P, incorporation into rRNA and 5S RNA was severely reduced while the amount into tRNA was not affected. The continuing incorporation into tRNA at 40 C appeared not to be due to terminal addition (CCA) based upon the base ratio of ^{32}P-labeled RNA and the incorporation of [^{3}H]uridine. Although a novel RNA (0.49 x 10^{6} daltons) was produced as rRNA and 5S RNA synthesis were reduced, hybridization experiments demonstrated that this RNA does not have sequences common to rRNA, 4S, or 5S RNA.

The origin and function of the 0.49 X 10^{6} dalton RNA produced in heat-stressed plants is not known. One possibly is that it is a derepressed mRNA

similar to those occurring in Drosophila during heat stress. However, this is not supported by the protein synthesis profile at elevated temperatures. Whatever the origin and function of this RNA, its synthesis specifically can be manipulated with heat treatments and should serve as a useful tool to examine mechanisms of gene regulation in plants. Additionally, the ease of obtaining large amounts of labeled 0.49 X 10^6 dalton RNA should make it useful in identifying and mapping restrictive fragments of plant DNA.

REFERENCES

1. Tissieres, A., Mitchell, H.K. and Tracy, U.M. (1974) J. Mol. Biol. 84, 389-398
2. McKenzie, S.L., Henikoff, S. and Meselson, M. (1965) Proc. Nat. Acad. Sci. USA 72, 1117-1121.
3. Lewis, M., Helmsing, P.J. and Ashburner, M. (1965) Proc. Nat. Acad. Sci. USA 72, 3604-3608.
4. Spradling, A., Pardue, M.L. and Penman, S. (1977) J. Mol. Biol. 109, 559-587.
5. Henikoff, S. and Meselson, M. (1977) Cell 12, 441-451.
6. Biessmann, H., Levy, W.B. and McCarthy, B.J. (1978) Proc. Nat. Acad. Sci. USA 75, 759-763.
7. Compton, J.L. and McCarthy, B.J. (1978) Cell 14, 191-201.
8. Dawson, W.O. (1976) Virology 73, 319-326.
9. Petrovic, S.L., Sumonja, B.D. and Vasiljevic, R.B. (1974) Biochem. J. 139, 157-162.
10. Kang, C-Y., and Temin, H.M. (1974) J. Virol. 14, 1179-1188.
11. Zartlin, M. Duta, C.T. and Petti, M.A. (1973) Virology 53, 300-311.
12. White, J.L. and Dawson, W.O. (1978) Virology 88, 33-43.
13. Siegel, A., Hari, V. and Kolacz, K. (1978) Virology 85, 494-503.
14. Laskey, R. and Mills, A. (1975) Eur. J. Biochem. 56, 335-341.
15. Aviv, H. and Leder, P. (1972) Proc. Nat. Acad. Sci. USA 69, 1408-1412.
16. Key, J.L. and Silflow, C. (1975) Plant Physiol. 56, 364-369.