GENETIC INFORMATION IN CHLOROPLAST DNA

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The structural organization and conformation of chloroplast (ct) DNAs from higher plants have been reviewed by Tewari et al.[1] and Bedbrook and Kolodner[2]. The ctDNA from higher plants exists as covalently closed circular DNA molecules with significantly different sizes that range from $85.4X10^6$ for corn ctDNA to $97.2X10^6$ for lettuce ctDNA[3]. The genetic information contained in ctDNA has been largely studied by molecular DNA-RNA hybridizations. Such studies have shown that ctDNA codes for ribosomal RNA (rRNA), transfer RNAs (tRNA), and messenger RNAs (mRNA). Thomas and Tewari[4] have reported two rRNA genes in the ctDNA of higher plants. Bedbrook et al.[5] have mapped the rRNA genes in restriction endonuclease map of the corn ctDNA. Hybridization experiments between ctDNA and ct tRNAs have revealed the presence of 40, 42, and 27 tRNA genes in pea, spinach and corn ctDNAs, respectively[6]. The amount of ctDNA involved in the coding of rRNA and tRNAs is only about $5X10^6$ daltons, whereas mRNA transcripts are coded by the remaining $45X10^6$ daltons of ctDNA. At saturation hybridization, 50% of the ctDNA hybridized to the in vivo labelled RNA, suggesting thereby the transcription in vivo of RNA molecules equivalent to a complete single strand of ctDNA[7].

The presence of both poly(A^+) and non poly (A^-) containing RNA transcripts of ctDNA in chloroplasts has been reported[8]. About 65% of the poly (A^+) RNA isolated from RNase treated chloroplats hybridize with corn ctDNA. These hybridizations represent the mass of poly (A^+) RNA hybridizing to ctDNA. Oishi et al.[9] have found that 20-25% of the pea ctDNA codes for poly (A^+) RNA transcripts.The mRNA transcripts of the ctDNA in pea range in sizes from

0.3 to $3X10^6$, and poly (A^+) tracts in ctDNA specific RNA range from 50 to 150 adenosine residues.

The present communication is aimed at finding out whether there occur any changes in mRNA transcripts in ctDNA of higher plants during their evolution.

Material and Methods

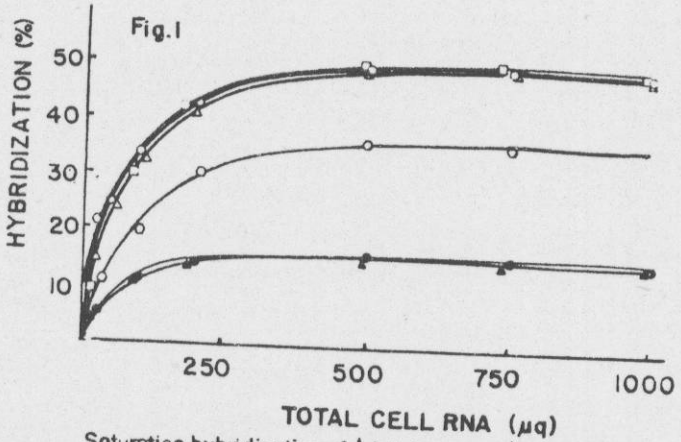
Pea and spinach, representing dicots, and corn from amongst monocots were used as the experimental material for the present studies. Pea and corn were grown in a glass house under natural light conditions at 28°C. Fresh spinach leaves obtained from the market and 7-8 days old pea and corn seedlings were used for experiments.

i) RNA extraction: 50 g of leaves were homogenized in chilled 100 mM Tris buffer (pH 7.5) and 0.1% diethyl pyrocarbonate. The homogenate was filtered through four layers of cheese cloth and four layers of mira cloth. The density of the solution was adjusted to 1.70 g/ml by adding solid CsCl. The homogenate was then centrifuged in a SW 27 rotor for 48 h at 20 K at 20°C. The RNA pellet was dissolved in 50 mM Tris, 25 mM KCl, 25 mM $MgCl_2$, 2% SDS (pH 7.5) and was extracted two times with water saturated phenol and two times with chloroform:isoamyl alcohol (98:2). RNA was collected by ethanol precipitation and redissolved in 0.15 M NaCl, 2 mM EDTA (pH 7.5).

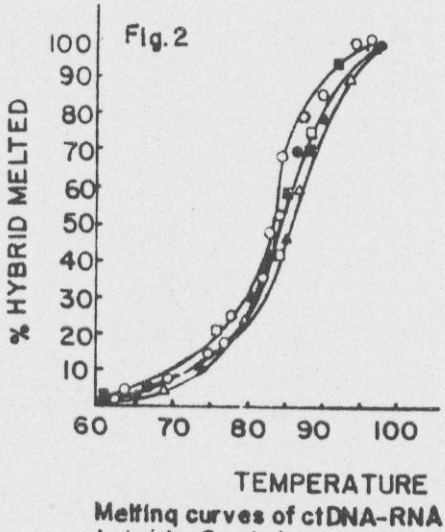
ii) 'Nick translation' of ctDNA: The ctDNA was isolated by the method of Kolodner and Tewari[10]. The 'nick translation' of ctDNA was carried out by the method of Maniatis et al.[11] with slight modifications. 7.5 µg of ctDNA was incubated in 200 µl at 18°C for 15 min with 2.5 µg of DNase in the presence of BSA (1 mg/ml), $MgCl_2$ (7.5 mM), and Tris (50 mM, pH 8.0). After incubation the reaction mixture was heated at 70°C for 10 min in a water bath. 150 µl of the above reaction mixture was incubated at 16°C for 90 min in the presence of β mercaptoethanol (10 mM),

0.5 µg of dATP, dGTP, dCTP, ^{32}P dTTP (50 c/m/mole), Tris (50 mM), $MgCl_2$ (10 mM) and 1 unit of DNA polymerase (Boehringer and Mannheim). The volume of the reaction mixture was made to 200 µl. The reaction was stopped by the addition of SDS to make 1% final concentration. The polymerized product was extracted with phenol saturated with 100 mM Tris (pH unadjusted). The aqueous phase was filtered through Sephadex G-100 column equilibrated with 0.15 M NaCl, 2 mM EDTA. The fractions showing maximum radioactivity were pooled and adsorbed to a hydroxyapatite column at 60°C in 0.12 M phosphate buffer. The column was thoroughly washed to bind only double stranded DNA. The column was then heated to 100 C and single stranded DNA was eluted in 0.12 M phosphate buffer. The DNA was incubated at 60°C for 30 min to renature snap back DNA and passed through hydroxyapatite column. The process was repeated two more times so as to get rid of hair pin loops. The radioactive DNA thus obtained was 99% susceptible to S_1 nuclease (Boehringer and Mannheim). The 'nick translated'DNA showed 96% renaturation when incubated with unlabelled ctDNA. The Cot 1/2 of the 'nick translated'DNA was undistinguishable from the Cot 1/2 of the unlabelled ctDNA.

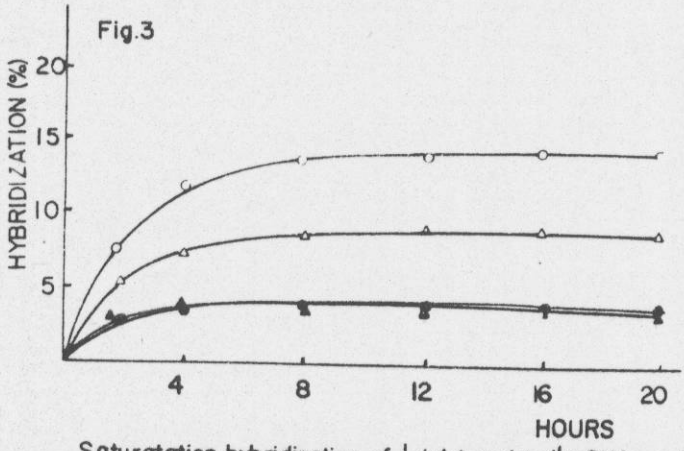
iii) <u>DNA-RNA hybridization</u>: The 'nick translated' DNA was hybridized to RNA in 1XSSC at a final volume of 200 µl at 65°C for 18 h. The control contained calf liver RNA in the same concentration as that of plant RNA. After incubation 10 µg each of single and double stranded calf thymus DNA, 0.3 M NaCl, 2 mM $ZnSO_4$ and 30 mM Na Acetate (pH 4.6), and S_1 nuclease was added and the volume of the reaction mixture was made to 1 ml and incubated for 2 h at 45°C. The amount of S_1 used in these experiments was enough to chew up 99% of the single stranded DNA. After the incubation period, 10 µg of RNase and 5 units of T_1 were added to the reaction mixture and incubated for 1 h at 37°C. Only stable DNA-RNA hybrids were left after these treatments which were precipitated by cold TCA (5% final concentration) in the



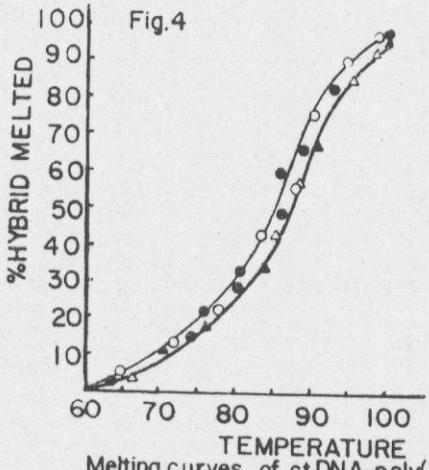
Saturation hybridization of 'nick translated' ct DNAs and total RNA of leaves. pea ct DNA-pea RNA(○), corn ct DNA-corn RNA(△), spinach ct DNA-spinach RNA (□), pea ct DNA-corn RNA(●), corn ct DNA-pea RNA(▲), pea ct DNA-spinach RNA (○).



Melting curves of ct DNA-RNA hybrids. Symbols same as in Fig.1



Saturatation hybridization of 'nick translated' ct DNAs and poly (A') RNA from leaves. pea ct DNA-pea poly(A') RNA (○), corn ct DNA-corn poly(A') RNA (△), pea ct DNA-corn poly (A') RNA (●), corn ct DNA-pea poly (A') RNA (▲).



Melting curves of ct DNA-poly(A')RNA hybrids. Symbols same as in Fig.3

presence of 25 ug of calf thymus DNA.

iv) Separation of poly(A^+) RNA: Poly(U)Sepharose column (Pharmecia) was used for isolation of poly (A^+) RNA. The total cell RNA from pea and corn was dissolved in 25% formamide, 0.7 M NaCl, 50 mM Tris, 10 mM EDTA (pH 7.5)and applied to poly(U)Sepharose column that was equilibrated and washed repeatedly in the same buffer. Bound RNA was eluted from the column with a buffer containing 90% formamide, 10 mM EDTA, 10 mM potassium phosphate (pH 7.5). The eluted RNA was diluted ten fold with loading buffer and passed through a fresh poly(U)Sepharose column. The whole process was repeated three times.

Results

Hybridization of ctDNA with total cell RNA: Varying concentrations of total cell RNA from pea, spinach and corn were hybridized to 0.05 ug of respective 'nick translated' ctDNAs. The data in Fig.1 show that increasing the concentration of RNA up to 250 ug increased the level of hybridization which reached a plateau at about 500 ug RNA. Increasing the concentration of RNA up to 1 mg did not increase the hybridization. At saturation, 50% of ctDNA hybridized to total cell RNA. The 'nick translated' ctDNA, when hybridized to corn total cell RNA, showed about 15% hybridization at saturation. Almost the same level of hybridization was obtained using the'nick translated' corn ctDNA and pea total cell RNA. At saturation hybridization it was found that about 35% of the pea ctDNA had base sequences common to spinach total cell RNA. Further experiments using 'nick translated' spinach ctDNA are in progress and would be described later.

Specificity of ctDNA-RNA hybrids: This was tested by competition experiments and thermal stability. The hybrids were stable up to 70°C (Fig.2). On increasing the temperature they melted with a sharp Tm of about 84°C.

Hybridization of ctDNA with poly (A^+) RNA: The 'nick translated' pea ctDNA showed about 15% hybridization with pea poly (A^+) RNA and nearly 2.5% with corn poly (A^+) RNA (Fig.3). At saturation hybridization, 6% of the corn ctDNA was found to hybridize with corn poly (A^+) RNA and about 2.5% of corn ctDNA had base sequence complementary to pea poly (A^+) RNA. The thermal stability of the ctDNA-poly (A^+) RNA confirmed the specificity of these hybridizations (Fig.4).

Hybridization of ctDNA with poly (A^-) RNA: The level of hybridization obtained with poly (A^-) RNA and respective ctDNA was the same as obtained with total RNA, i.e., 50% of the ctDNA was found to hybridize with poly (A^-) RNA. With heterologous systems also, similar results were obtained as in the case of total cell RNA. The thermal stability analyses of the hybrids showed the specificity of the base pairing.

Discussion

The saturation hybridization data of ctDNAs and respective total cell RNAs showed that one strand equivalent of ctDNA is transcribed into RNA. In heterologous systems using 'nick translated' pea ctDNA and hybridizing it with total cell RNA from spinach and corn, about 35% and 15% hybridization was obtained, respectively. Similarly, with 'nick translated' corn ctDNA and pea RNA about 15% hybridization was recorded at saturation. The above findings suggest a closer base sequence homology between pea and spinach ctDNAs (both of which are dicots) as compared to that between ctDNAs of pea and corn. In the latter case about 30% base sequence homology was observed. These results were also confirmed by hybridizing 'nick translated' corn ctDNA and total cell RNA from pea. The hybrids between ctDNAs and total cell RNAs were formed by specific base pairing since the Tm was found to be the same as that of respective native ctDNAs. Lamppa and Bendich[12] investi-

gated the base sequence homology between 'nick translated' pea ctDNA and total cellular DNA from a number of higher plants and in each case the probe reassociation was found accelerated suggesting that some ctDNA sequences have been highly conserved throughout the evolution of vascular plants. However, they did not compare the sequence homology between ctDNAs of various plants.

The saturation hybridization studies with pea ctDNA and *in vivo* labelled pea ctRNA have shown that 50% of the ctDNA was transcribed *in vivo*[9]. It was also reported that the transcription of pea ctDNA was sufficient to code for about 80 polypeptides of average molecular size of 50,000 assuming that 1.5 kilo bases of DNA constitute one gene. It was further reported that there existed a difference in the transcription of ctDNA in etiolated and light grown plants to the extent of only 2% of the ctDNA, which could code for 3-4 genes that were not transcribed in the dark. Bedbrook *et al.*[13] reported the presence of plastid gene in maize ctDNA which was expressed during photoregulated plastid development. Developing plastids and mature chloroplasts, but not etioplasts, contain mRNA that hybridized to Bam fragment 8 of ctDNA and was translated *in vitro* into a 34,500 daltons protein. Chelm and Hallick[14] reported the hybridization of 'nick translated' DNA to total cellular RNA isolated from *Euglena* at various stages of chloroplast development. The level of transcription of ctDNA in dark adapted cells was 17%, which decreased in the initial stages of plastid development and subsequently increased to 23% at the end of 72 h of light growth.

Oishi *et al.*[9] have found that about 0.18% of the pea RNA represents poly (A^+) RNA which hybridized with 20-25% of the pea ctDNA. In contrast to reports on viral and eukaryotic organisms where almost all mRNAs contain poly (A^+) tracts[15], pea and corn ctDNAs contain a small proportion of poly (A^+).

In the present studies about 15% hybridization was obtained at saturation hybridization of 'nick translated' pea ctDNA and poly(A^+) RNA from whole cell. The corn poly (A^+) RNA was found to hybridize with 6% of the corn ctDNA. This is consistant with the findings of Haff and Bogorad[8] However, with heterologous system using 'nick translated' pea ctDNA and corn poly (A^+) RNA, and 'nick translated' corn ctDNA and pea poly (A^+) RNA around 2.5% hybridization was recorded. The hybridization of ctDNA with poly(A^-) RNA has shown that all of the mRNA species which contain poly (A^+) were also found in poly (A^-) RNA. It is not known whether poly (A^+) RNA transcripts which are common to both pea and corn are transcribed from a specific region of the genome or the genes for these transcripts are distributed throughout.

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