STRUCTURE OF A HEAVILY TRANSCRIBED REGION OF BARLEY CHLOROPLAST DNA. TRANSFER RNA GENES FOR SERINE (UGA), GLYCINE (GCC,UCC), FORMYL-METHIONINE AND THREONINE (GGU)

by

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The barley chloroplast genome is transcribed into at least 80 RNA molecules (29). Some regions of the genome hybridise to more transcripts than others. One 4.3 kbp HindIII fragment was found to hybridise to a disproportionately high number of transcripts, some of which were light-induced. The sizes of the transcripts ranged from 0.3 to 5.5 kb. In order to discover from which genes these transcripts are derived, the DNA sequence of the fragment has been determined and the transcripts have been mapped. Five tRNA genes have been located including genes for formyl-methionine tRNA and a glycine tRNA with an intron in the D-stem. A scheme to account for the transcription and processing of a precursor-transcript for these tRNAs is proposed. A region containing two open-reading frames hybridises to 9 transcripts. The largest of these, which is 5.5 kb, may be intercritronically processed. A number of long repeated sequences which may be involved in recombinational activity were found.

1. INTRODUCTION

Chloroplasts contain multiple copies of a circular DNA molecule of about 115 to 180 kbp (45). The genome of barley is typical of many higher plants. It is about 135 kbp and contains inverted repeats of 21 kbp (29). In an earlier study, more than 80% of the genome was cloned in bacterial vectors (29). Selected DNA fragments which had been isolated and nick-translated, were hybridised to electrophoretically separated filter-bound chloroplast RNA. This study revealed more than 70 discrete RNA transcripts of the genome. The transcripts were

not distributed evenly around the genome. One 4.3 kbp HindIII fragment, cloned in the plasmid pHvC35, was found to hybridise to at least 10 transcripts ranging from 300b to more than 5kb. Moreover, at least 4 of these were found to be more abundant in plastid RNA isolated from plants illuminated for 8 hours, than in etioplast RNA. This indicated that these transcripts were light-induced.

The disproportionately heavy transcription of this region prompted the present study. The DNA sequence has been determined and the transcripts mapped. Five tRNA genes have been

Abbreviations: b = base; kb = kilo base; kbp = kilo base pairs; ORF = open reading frame; pHvC = recombinant plasmid containing a barley chloroplast DNA fragment; trnX-YZZ = tRNA gene for amino acid X, anticodon YZZ

Figure 1. Summary of the DNA sequence analyses and transcript mapping of pHvC35. The horizontal scale represents the 4294 bases of the sequence. Green arrows = open-reading frame; red arrows = tRNA genes; black arrow = repeated sequence. The vertical scale indicates the size of constitutive transcripts (dark-blue) and light-induced transcripts (light-blue). The M13-probes used for transcript mapping are shown just above the abscissa.

identified. These include genes for the initiating methionine tRNA and for a glycine tRNA with an intron in the D-stem. Sequences corresponding to the 3' portion of a long open-reading frame (ORF) hybridise to 9 transcripts. This multiplicity of colinear transcripts indicates extensive processing of the primary transcript. A number of tandem and inverted repeats of 16-40 b were found. This may indicate that this region of the plastid genome is active in recombination (3, 11, 42).

Preliminary data and summaries of this work have appeared elsewhere (31, 32, 44).

2. MATERIALS AND METHODS

2.1. Nucleic acids

DNA of the plasmid pHvC35, which contains a 4.3 kbp HindIII fragment inserted in the tetracycline resistance gene of pBR322, was prepared according to BIRNBOIM and DOLY (2).

M13mp8 and M13mp9 (24) replicative forms were gifts from S.K. RASMUSSEN.

Plastid RNA was prepared from etioplasts and chloroplasts by lysis in guanidinium thiocyanate and centrifugation through a cushion of CsCl (29). In some experiments plastids were extracted with phenol and chloroform and the nucleic acid precipitated directly. These preparations contained DNA and small mol. wt. RNA as well as large mol. wt. RNA.

2.2. DNA sequencing

Restriction endonuclease digests (HindIII, Sall, BamHI, EcoRI, PstI, XbaI, EcoRI', RsaI, HpaI, TaqI and Sau3A) of pHvC35 or its subclones were ligated randomly into the appropriately cleaved replicative form of M13mp8 or M13mp9 (24). After transfection into *E. coli* JM101 recombinants were propagated and sequenced as described (34, 35). Extra data were

Figure 2. Physical map of barley chloroplast DNA showing the location and orientation of pHvC35 DNA. The cleavage sites of the restriction enzymes used in the sequence analysis are indicated. Only one small BamHI fragment was digested with EcoRI*. Black arrows represent the direction and extent of sequence information obtained from each M13 subclone.

obtained from one clone which contained a SalI fragment corresponding to nucleotides 2294 to 1598 in the sequence (Figure 2). A 168 bp XbaI fragment (nucleotides 2204 to 2036) was removed by digesting replicative form DNA with XbaI and electrophoresis on a low-melting point agarose gel. The large fragment was purified, ligated, transfected and sequenced. Sequence data were analysed using a Honeywell Bull computer and the programs of Dr. R. STADEN (MRC, LMB, Cambridge, UK).

2.3. Transcript mapping

Chloroplast RNA was electrophoresed in 1.5% Seakem agarose gels containing formaldehyde as before (29) and blotted onto Genescreen filters (New England Nuclear, Mass) as described by the manufacturers. Filters were pre-hybridised overnight in plastic bags containing $0.2 \text{ ml} \cdot \text{cm}^{-2}$ of a solution containing 50% (v/v) de-ionised formamide, 0.2% (w/v) bovine serum albumin, 0.2% (w/v) ficoll 40000, 0.2% (w/v) polyvinylpyrrolidone, 1% (w/v)-Na-dodecylsul-

10	20	30	40	50	60
AAGCTTCTCA	AGCTCAAGCA	TTTACTTTTC	TAGTTAGAGA	CCAGCGTCTT	GGAGCTAATG
70	80	90	100	110	120
TGGGATCCGC	TCAAGGACCC	ACAGGTTTTC	GGAAATATCT	AATCCGTTCC	CCAACTGGGG
130	140	150	160	170	180
AGGTTATCTT	TGGAGGGGAA	ACTATGCGTT	TTTGGGACCT	TCGTGCTCCA	TGGTTAGAAC
190	200	210	220	230	240
CTCTAAGGGG	CCCCAACGGT	TTGGACTTGA	GTAGGTTGAA	AAAAGACATA	CAACCTTGGC
250	260	270	280	290	300
AAGAACGGCG	CTCAGCAGAA	TATATGACCC	ACGCTCCTTT	AGGCTCTTTA	AATTCCGTGG
310	320	330	340	350	360
GTGGCGTAGC	GACCGAGATC	AATGCAGTTA	ATTATGTCTC	TCCTAGAAGT	TGGTTATCAA
370	380	390	400	410	420
CTTCTCATTT	TGTTCTAGGA	TTCTTCTTTT	TTGTGGGCCA	TTTATGGCAT	GCAGGAAGAG
430	440	450	460	470	480
CCCAGAGCTG	TGCAGCAGGT	TTTGAAAAGG	GAATCGATCG	TGATTTGGAA	CCTGTTCTTT
490	500	510	520	530	540
ACATGAACCC	TCTTAACTAA	GATTTTCTTA	TTTATACCTG	TTCTACTTTG	TTTCTGTCTT
550	560	570	580	590	600
GGCTCGGTTA	TTCTATCTAG	CCGAGCCATT	CATTCCTTTT	TATGAAAGAA	AGATAAGGGA
610	620	630	640	650	660
CAGAATAAAA	AAAAATGAAA	GAAACAAACG	TATTCAATAA	GCAAAAGGAG	AGAGAGGGAT
670	680	690	700	710	720
TCAACCCCTC	GATAGTTCCT	AGAACTATAC	CGGTTTTCAA	GACCGGAGCT	ATCAACCACT
730	740	750	760	770	780
CAGCCATCTC	TCCACCGCCT	AATCCTTATT	TTACTCCTAC	AAATAGAACA	TAGCCATATA
790	800	810	820	830	840
AAAAATAAAA	ATATTTATTA	ACCTCTAGAA	AGATATCAGA	TACAAGTTCC	TTTTCGATAT
850	860	870	880	890	900
ATCTCTGTAT	ACTGTATACA	CGGATACAGG	ATCCGCTATA	CCCCTTGTG	AAATAAGAGC
910	920	930	940	950	960
TAAATAACCT	CCTCTCACCC	CCATATCCAA	ATAAAAAAGA	GGTTAAGTAA	TAAATTTTAA
970	980	990	1000	1010	1020
TTAAAGAGAA	GAATCAATGG	ATTTCATGATT	AAACCCCTCC	TACTTCTTGT	ATTTTTTTAC
1030	1040	1050	1060	1070	1080
AATTTTGGTT	AAGTGAGGGA	TCAAATATGT	AGTCAACTTT	ATTTGATGGT	AGCTTGGAGG

Figure 3. DNA sequence of pHvC35. Only the 5'-3' direction is shown.

1090	1100	1110	1120	1130	1140
ATTAGAAATA	TGACTATTGC	TTTCCAATTA	GCTGTTTTTG	CATTAATTGC	GACTTCCTCA
1150	1160	1170	1180	1190	1200
GTCTTAGTAA	TTAGTGTACC	CCTTGATTAT	GCTTCTCCTG	ATGGTTGGTC	AAATAATAAA
1210	1220	1230	1240	1250	1260
AATGTTGTAT	TTTCCGGTAC	ATCATTATGG	ATTGGACTAG	TCTTTCTCGT	AGCTATTCTG
1270	1280	1290	1300	1310	1320
AATTCTCTCA	TTTCTTAAAT	TAAATTTGGT	TAATATTTAG	TAACCCCAT	AAAAAGAAAT
1330	1340	1350	1360	1370	1380
AATAAAGGCC	ATTTAAAAAA	TTCGAATAGT	GAGACGTATT	AAAACGCAAT	TTGCGTTCCG
1390	1400	1410	1420	1430	1440
AATTGCTAGC	TCTTCTCTTT	CAGTATTATG	AAATTCCCAT	TAGAATATTC	ATTGACAGAT
1450	1460	1470	1480	1490	1500
AATAAAAAAA	GGAAAACCTCT	AATATCTAAT	ATAATAGAAT	AGAAAATGAA	ATGAAACGGT
1510	1520	1530	1540	1550	1560
CGACCCAGAC	ATAGACGGTC	GACCCAAGCG	GATATACGCT	ATAAAATATA	TACCGTAGCG
1570	1580	1590	1600	1610	1620
AGCGTAGTTC	AATGGTAAAA	CATCTCCTTG	CCAAGGAGAA	GATACGGGTT	CGATTCCCGC
1630	1640	1650	1660	1670	1680
CGCTCGCCCG	CTTAATTTAT	CAAAGTACTA	TGATAAAAGT	TTAGTCTAGT	TAATTGTTTA
1690	1700	1710	1720	1730	1740
ACTACTTATC	TTAAATTAAG	TATTAATTAA	GTATTAGTCT	AGTGCCGTAT	CCCTTACTAT
1750	1760	1770	1780	1790	1800
ATTTACCCTT	CCTTTGCATC	CCCCTCAAAA	AAAGAGGGGG	CTCCGGAGGC	AGAAATAGAA
1810	1820	1830	1840	1850	1860
CTAGCCAACA	GAGCCCCAAA	ATTGGGTATT	AACTGAGACA	AACAACGGGC	AAACTGTTTT
1870	1880	1890	1900	1910	1920
TAAAAGGAAG	GGAGAGGAGA	CTGTCCCTTT	CATTTCATTT	TTATTTTCTG	CAAGGTAGGG
1930	1940	1950	1960	1970	1980
AGAAGCCTTG	CGCCTTCTTT	TTGTGAAGGC	AAGTGGCTTC	GCAAATTGCT	CAATTTTGCC
1990	2000	2010	2020	2030	2040
CTCTAGGGCC	CGGAACATAA	TAATAAAAAA	GGGTGGGATA	CGCCCTCTA	CCATATCTAG
2050	2060	2070	2080	2090	2100
AGAAATAGAA	TACTCCTTTT	ATAGAGACTG	CTAAGTGCGG	AGACGGGAAT	CGAACCCGTG
2110	2120	2130	2140	2150	2160
ACCTCAAGGT	TATGAGCCTC	GTGAGCTACC	AAACTGCTCT	ACTCCGCTCT	GTAGTACCAG

2170	2180	2190	2200	2210	2220
AAACTGGTGG	ACAAAAAAGG	CTTGAATACA	AGCCTTTAAC	ATGTCTAGAC	AAATAGAATA
2230	2240	2250	2260	2270	2280
CTCCTTTTAT	ACAGAATGGA	GCGGGTAGCG	GGAATCGAAC	CCGCATCGTT	AGCTTGGAAG
2290	2300	2310	2320	2330	2340
GCTAGGGGTT	ATAGTCGACG	TTGGTTGATT	ATTTTAAACG	TCTCTAATTC	AAAACCGAAC
2350	2360	2370	2380	2390	2400
ATGAAATTTT	GATTTTCATC	GGCTCCTTTA	TGGATATTCT	CACCACTTAA	CATCTATGTC
2410	2420	2430	2440	2450	2460
AGCTTTTCTA	TTTGAATGGA	ACCAAAGCTC	TCTGCTTTCT	AGATGATCCT	TATAGAGTAG
2470	2480	2490	2500	2510	2520
GAGATAGAAA	TTCTATCTAA	ATCCATCTAA	TCTACTTACT	TCGTTCCCTA	ATTTTCATCA
2530	2540	2550	2560	2570	2580
AGAGATCCTG	AGGAAAAGAA	TTGGGTTTCC	ACCGAGCTGA	AACAATATGC	GGATGGTTCT
2590	2600	2610	2620	2630	2640
AGTAAACCAA	AACTATCGTT	TTTTAGCTAT	TTGGCTTCCT	TATCCTTTAA	CAAAAGAAGA
2650	2660	2670	2680	2690	2700
TTTAGTTACG	ATTGGAAATC	AACTTTTTTG	TATCTTCATC	CATAGATCCT	TTACTCATAT
2710	2720	2730	2740	2750	2760
TTTCAAAAT	GGAATACTTA	AATCCAATGC	AAAATTACCC	TTCCCGACTC	TGTACTCATA
2770	2780	2790	2800	2810	2820
TCATAATCCA	ATTTTTATTT	TGGATGCAAT	TTAAATTAGT	TTTTGGGTAC	AAATCGCCAG
2830	2840	2850	2860	2870	2880
AATGTATATT	CTTCCTCAAT	ATGCTATTGA	GAGGAAACGG	ATTTAATCCT	TTATAAGAAC
2890	2900	2910	2920	2930	2940
TAAAGTTTTC	ATCGGAATAT	AAAAAACCTT	AAGGATGCCT	TAAGTATATC	ATTTCAAATT
2950	2960	2970	2980	2990	3000
CAGTTATTAA	TAGAACGAAT	CACACTTTTA	CCACTAAACT	ATACCCGCTA	CATTGTATAT
3010	3020	3030	3040	3050	3060
TATGGTATAA	ATGGTACCCT	TTGTCAAGGA	TAGCCATTTG	ACAAGGAGGC	TAATTCCCCC
3070	3080	3090	3100	3110	3120
TTATTGAATC	AGATGGAGAA	GGTTCATGAC	ACTGAGCCAT	TGGTACTTCT	ACTTCGATCG
3130	3140	3150	3160	3170	3180
CGCGCCTTTA	CTTTAGGATT	TAGATAGCCC	CTCTCCCTCT	GGTCTGTAAA	ATAAATCTCT
3190	3200	3210	3220	3230	3240
TCTTACCCTG	CTAATAGCGC	CTGAACCAAA	TGTATTTATT	TTGATTAGGA	TCCTATCCTA

3250	3260	3270	3280	3290	3300
TTCTACGGTT	ACGACTACAA	TAATCATTAT	TTACTTTGCG	AGGACGTAAG	TGTGCCAGAC
3310	3320	3330	3340	3350	3360
TGCTAAATGG	TTTATTATTT	ATTCCTACAA	TATAAACTAG	GGGATATAGG	GGGTAACACT
3370	3380	3390	3400	3410	3420
CTCCCTATAA	ATTCCTTTTT	TCCTTTTCTT	TTTGTGCCAG	AATTGAACAA	AAAAGAAATT
3430	3440	3450	3460	3470	3480
ATAGAAGATG	TTTCTTCC	CCATTAAGTC	CGAGCCATAG	AGTAAGAGGT	GAGATGAGTT
3490	3500	3510	3520	3530	3540
TTCCAAATTC	ATCATAGACT	TTCCCTATGG	CTTGATAGAA	GTAAGAAGCA	AAGAGTTGTA
3550	3560	3570	3580	3590	3600
ACTTAAAGAG	TCGTAACCTTA	AAGAAAAAAA	CGGACAGGGC	TGACAGATTT	ACCTGATGTA
3610	3620	3630	3640	3650	3660
AAGAAGATCC	TAAAAGTCTA	TGATTAGTCG	ACTCTCTCCA	TTTAACACTT	TCCTCTCTCT
3670	3680	3690	3700	3710	3720
CCTTTTTTCC	ACTGACTCAA	TGCTAGTTTA	TTAGATTCTT	GTTTAAAAGA	ATCAAAAAAG
3730	3740	3750	3760	3770	3780
TTCAATAGAA	CTAAGAACAC	ATAAAAAATA	GCATAGAGGA	TCAAGGCCAT	TACCAAAAGT
3790	3800	3810	3820	3830	3840
TCCTCCCAAG	GATCCTATCC	TATTGAGTAT	CTGTTCCCTG	CCTTTCCCGT	AGGTTAGGAA
3850	3860	3870	3880	3890	3900
ATCTTATATT	TTCCATATAC	CATTGAATCC	TTGGGGTTCC	AGAATCCGCC	TATTTTACTA
3910	3920	3930	3940	3950	3960
GCCTTCGGAA	ATCGGAAAAC	CATGAACCAA	GAAGAGTTGG	GTAGTTTTCT	TTCCCTAATT
3970	3980	3990	4000	4010	4020
TTATTTTGAG	CTCTCAAGAT	ATCAATACAT	ATACAATCTA	TACATAATGT	CTCTCTCTAT
4030	4040	4050	4060	4070	4080
ATCTCTATAT	ATTATATATA	TATAATATGT	ACATTATGCA	GTAGACCCAT	AATGGGAAAT
4090	4100	4110	4120	4130	4140
CAAAGTGGA	AATTTTGTAA	TTAAATAAAA	AGCCCTTTTA	ACTCAGTGGT	AGAGTAATGC
4150	4160	4170	4180	4190	4200
CATGGTAAGG	CATAAGTCAT	CGGTTCAAAAT	CCGATAAAGG	GCTTTTTTAA	TTAGTGGTAG
4210	4220	4230	4240	4250	4260
AGTAATCTCG	TGCTAAGACG	TAAGTCATTG	GTTCTGAATCT	GATAGAGTAC	TTTCTACTA
4270	4280	4290			
AATTGATTCTG	CTTTTTTCTT	TGTTTTTGAA	GCTT

Figure 4. Northern analysis of the constitutive transcripts.

RNA from etioplasts (0) and 8-hour greened chloroplasts (8) was electrophoresed on agarose gels and blotted onto Genescreen. Four probes (63-874, 63-345, 342-874 and 1217-1648) were made radioactive and hybridised to the Genescreen filters as described in section 2.3.

phate, $0.5 \text{ mg} \cdot \text{ml}^{-1}$ sonicated, phenol-extracted, heat-denatured salmon sperm DNA, 0.75 M -NaCl and 75 mM -Na-citrate at 42°C with shaking.

Probes were prepared from M13 subclones annealed to either a sequencing primer (30) or the "hybridisation" primer (13) (both from Boehringer, Mannheim, FRG). In both cases the

primer was extended using 0.5 units DNA polymerase (Klenow subfragment), 10–50 μCi $\alpha^{32}\text{P}$ -dATP (600 $\text{mCi} \cdot \text{mmol}^{-1}$, New England Nuclear), 50 μM -dGTP, 50 μM -dCTP, 50 μM -dTTP, 50 mM -NaCl, and 10 mM -Tris-HCl, pH 8.0 in a total volume of 20 μl . The mixtures were incubated at either 15 $^{\circ}\text{C}$ for 90 min (hybridisation primer) or room temperature for 15 min (sequencing primer). When using the sequencing primer, the reaction was chased for a further 15 min with 100 μM -dATP. Thereafter the double-stranded DNA was cut with HindIII (M13mp9 subclones) or EcoRI (M13mp8 subclones). Unreacted dATP was removed from both types of probe by passage through a Sephadex-G50 column equilibrated in 10 mM -Tris-HCl, pH 8.0, 1 mM -EDTA.

After removal of the prehybridisation solution from the plastic bags, the hybridisation solution (as for prehybridisation but 0.1 $\text{ml} \cdot \text{cm}^{-2}$ solution and 250 $\mu\text{g} \cdot \text{ml}^{-1}$ salmon sperm DNA were used) containing 10^6 – $5 \cdot 10^6$ cpm of probe was added and shaking was continued for 40 hours. The probes produced with the sequencing primer were boiled for 5 min before use.

The filters were washed twice with both 0.3 M -NaCl, 30 mM -Na-citrate, 0.1% (w/v) Na-dodecylsulphate at room temperature and with 15 mM -NaCl, 1.5 mM -Na-citrate, 0.1% (w/v) Na-dodecylsulphate at 50 $^{\circ}\text{C}$. The filters were vigorously shaken for 15 min in trays containing about 150 ml of the solutions. Subsequently the filters were air-dried and autoradiographed. The rRNAs served as internal standards for the extrapolation of the sizes of the transcripts.

The endpoints of selected transcripts were determined by S1-nuclease digestion of hybrids between chloroplast RNA and radioactive single-stranded DNA probes (1). End-labelled

Figure 5. S1-mapping the 3' ends of the constitutive transcripts.

3'-end-labelled probe 2298-1522 was prepared and hybridised either to no RNA (-RNA), etioplast RNA (0h RNA), RNA from 6-hour greened chloroplasts (6 hours RNA) or RNA from 8-hour greened chloroplasts (8h RNA). Each sample was digested with S1-nuclease and electrophoresed on a 6% polyacrylamide gel. Undigested probe (3' 2298-1522) and a dideoxy-sequencing reaction were run as markers.

Figure 6. Northern analysis of the light-induced transcripts.

RNA was electrophoresed and blotted (2.3) and hybridised with the five indicated M13 probes. The filters d and e were obtained from a gel electrophoresed for a longer time than used for the filters a, b and c.

probes were prepared by digestion of pHvC35 with Sall, XbaI, XbaI + BamHI and Sall + Sau96-I. The fragments were labelled at the 5' end using polynucleotide kinase or at the 3' end using the fill-in reaction of the Klenow fragment of DNA polymerase (23). Double-stranded DNA was dissociated and the strands were separated on polyacrylamide gels and purified according to MAXAM and GILBERT (23). The single-stranded probes were hybridised to chloroplast RNA, digested with S1-nuclease and

electrophoresed on polyacrylamide gels using a dideoxy-sequencing reaction as size-marker as before (30).

2.5. Chemicals and enzymes

All chemicals were of Analar grade and obtained from sources described previously (29, 30). DNA modifying enzymes were from Boehringer, Mannheim, FRG except for Sall and DNA ligase (from New England Biolabs) and

polynucleotide kinase (from Amersham International, Bucks, UK.). All enzymes were used according to their manufacturers' instructions. Radioisotopes ($\alpha^{32}\text{P}$ -dATP, 600 Ci · mmol⁻¹ and $\gamma^{32}\text{P}$ -ATP, 3000 Ci · mmol⁻¹) were from New England Nuclear, Ma., USA.

3. OVERVIEW

Figure 1 presents an overview of the results and conclusions of this study. The horizontal axis represents the 4294 bases of the DNA sequence of the HindIII insert of pHvC35. The sizes and positions of open-reading frames (green arrows), transcripts (light-blue arrows for light-induced transcripts, dark-blue for constitutive transcripts), tRNA genes (red arrows) and short-repeated sequences (black arrows) are indicated. The sequence of a partially overlapping portion of maize chloroplast DNA (19, 21) has been similarly summarised.

4. RESULTS

4.1. DNA sequence of pHvC35

Figure 2 shows the location and orientation of the 4.3kbp HindIII fragment in the barley chloroplast genome in relation to the inverted repeats and the *rbcL* gene. The restriction endonuclease map of the fragment indicates the restriction fragments ligated into M13 vectors for sequencing. The arrows indicate the direction and extent of sequence information obtained from each subclone. In all but 4 places the sequence of both strands of the DNA has been obtained. Where not, the sequencing reactions were repeated.

The sequence obtained is shown in Figure 3. The 5'-3' direction of only one strand is shown. Locations within the sequence are referred to by quoting their inclusive coordinates. Fragments designated as having descending coordinates are from the strand complementary to the one shown. Coordinates of M13 subclones refer to the sequence complementary to their insert i.e. the sequence potentially obtained by the dideoxy-sequencing procedure.

4.2. Transcript mapping

4.2.1. Constitutive transcripts

RNA was isolated from chloroplasts, electro-

phoresed on formaldehyde/agarose gels and transferred to Genescreen filters. In some experiments RNA from etioplasts was co-electrophoresed in order to detect light-induced differences in the hybridising transcripts. Radioactive probes were made using M13 subclones of pHvC35. This approach allows the position, direction and size of a transcript to be estimated. The ends of each probe used are indicated in Figure 1. By using a number of overlapping probes the ends of the transcripts were mapped with a minimum accuracy of 300 bp.

Probe 63-874 hybridised to 9 transcripts of 1.8, 2.5, 3, 3.4, 3.5, 4, 4.4, 4.8, and 5.5 kb (Figure 4a). Artefactual hybridisation associated with the rRNAs (1.1 to 1.9 kb) has been ignored. No differences were observed between etioplast (0) and chloroplast (8) RNA. As expected probe 63-345 hybridised to the same 9 transcripts with similar intensities (Figure 4b). Likewise, probe 342-874 hybridised to all 9 transcripts (Figure 4c). However, in this case the hybridisation to four of the transcripts (1.8, 3.4, 3.5 and 4.8 kb) was proportionately weaker. This indicated that the 3' ends of these four transcripts were at about position 500 (Figure 1). This was confirmed by hybridisation using probe 1217-1648 (Figure 4d) which showed hybridisation to five transcripts (2.5, 3, 4, 4.4, and 5.5kb) without any trace of hybridisation to the other four. The same five transcripts hybridised to the large probe 869-3233 thus precluding additional large transcripts in the region beyond 1648 (data not shown).

A single-stranded 3'-end-labelled DNA probe 2298-1522 was hybridised to chloroplast RNA. After digestion with S1-nuclease, gel-electrophoresis revealed an S1-resistant fragment of 290 ± 1 bases (Figure 5). This indicates that the five transcripts hybridising to M13 probe 1217-1648 (Figure 4d) end close to position 1812.

4.2.2. Light-induced transcripts

Six light-induced transcripts were identified by Northern analysis. Judging by the relative autoradiographic intensities, each transcript was induced 3-6 fold by illumination for 8 hours.

Probe 3795-3228 (Figure 6a) hybridised to a transcript of 0.72 kb. This transcript did not

hybridise to probe 3632-2294 (Figure 6b). This indicates that the 3' end of this transcript lies between 3632 and 3795.

Probe 3233-1260 hybridised very strongly to transcripts of 0.62 and 0.86 kb and weakly to a transcript of 0.3 kb (Figure 6c). Artefactual hybridisation which appears to be associated with the rRNAs has been ignored. Probe 3632-2294 (Figure 6b) hybridised only to the 0.62 and 0.86 kb transcripts. Probe 2294-1518 hybridised only to the 0.86 kb transcripts and weakly to the 0.3 kb transcript (Figure 6c). The autoradiograms shown in Figures 6d and 6e resulted from an agarose gel that was electrophoresed for longer than normal. Hence the 0.3 kb transcript is much more diffuse than in Figure 6c. Taken together these results indicate that the 0.62 kb transcript fell between 3228 and 2294; the 0.3 kb transcript between 2294 and 1518; and the 0.86 kb between 3228 and 1518 (Figure 1).

Probe 3103-4054 hybridised to two transcripts of 1.8 and 0.8 kb (Figure 6e). There is a very heavy background in this autoradiogram which obscures the bands. Nevertheless, the presence of transcripts is supported by results of S1-nuclease analysis (Figure 7a). These transcripts were not mapped further by Northern analysis. Probes 1265-869 and 874-63 failed to hybridise to plastid RNA (data not shown).

The ends of some of the light-induced transcripts were mapped further using the Berk-Sharp technique (Figure 7). Hybridisation of 5'-end-labelled 3232-2442 to plastid RNA samples produced an S1-nuclease-resistant molecule of 159 ± 1 bases (Figure 7a). This indicates that either or both of the 1.8 and 0.8 kb tran-

Figure 7. S1-mapping the ends of light-induced transcripts.

a) 5' end-labelled 3232-2442, b) 5' end-labelled 2439-3229, c) 5' end-labelled 2036-2204, or d) 3' end-labelled 2299-1990 were hybridised to plastid RNA samples, digested with S1-nuclease and electrophoresed on polyacrylamide as described (2.3). 5' end-labelled 2036-2204 (c) was hybridised to etioplast or to chloroplast RNA prepared either by centrifugation through CsCl (O h-RNA-L, 8 h-RNA-L) or by direct phenol-extraction of plastids (O h-RNA-T, 8 h-RNA-T) and treated as before.

Figure 8. The non-coding strand of five putative tRNA genes folded into the cloverleaf structures. The numbers are the sequence coordinates.

scripts (Figure 6e) start at about position 3073. Judging by the autoradiographic intensity, the abundance of this (these) transcripts is low. Furthermore, RNA from 8 hour-greened chloroplasts protected significantly more of this fragment than did RNA from 6 hour-greened chloroplasts or from etioplasts. This confirms the result of the Northern analysis (Figure 6e) suggesting that the transcript was markedly light-induced.

Hybridisation of 5'-end-labelled 2439-3229 revealed a major S1-resistant fragment of 549 ± 3 bases together with weaker labelled fragments of 449 ± 3 and of 275 ± 2 bases (Figure 7b). This indicates start sites for transcripts at 2988 ± 3 , 2888 ± 3 , and 2714 ± 2 . Equal quantities of DNA probe were protected by each RNA sample. As Northern analysis (Figure 6c) had indicated that these transcripts were light-in-

duced, it is apparent that, in molar terms, there was excess transcript over DNA-probe during hybridisation, even when using etioplast RNA. This serves to confirm the remarkable abundance of these transcripts.

Using 5'-end-labelled 2036-2204 S1-resistant fragments of 113 ± 1 and 159 ± 2 bases were produced (Figure 7c). This indicates that the start of the 0.3 kb transcripts may be at about 2150 or 2195. No significant differences were observed when using RNA prepared without the CsCl-cushion and thus including small mol. wt. RNA.

A similarly complex picture emerged from the hybridisation of 3'-end-labelled 1990-2299 to plastid RNA (Figure 7d). Protected fragments of 61 ± 1 , 121 ± 1 , 132 ± 1 , and 299 ± 1 places 3' ends of transcripts at about 2238, 2178, 2167 and 2000. Some of the probe was protected along

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                -35 box                -10 box                =====
TTGTCAAATG GCTATCCTTG ACAAAGGGTA CCATTTATAC CATAATATAC AATGTAGCGG 2985
accac t c cc gt c a t gcatt g a -cgg t 4

=====5' exon=====
GTATAGTTTA GTGGTAAAAG TGTGATTCGT TCTATTAATA ACTGAATTG AAATGATATA 2925
tcccttaaat gt a aggg 61

CTTAAGGCAT CCTTAAGGTT TTTTATATTC CGATGAAAAC TTTAGTTCTT ATAAAGGATT 2865
tccttc gt tga tc t-- ---- -- c ac -ga tc a 113

AAATCCGTTT CCTCTCAATA GCATATTGAG GAAGAATATA CATTCTGGCG ATTTGTACCC 2805
t t g a g cga c ataca cgt t 174

AAAACTAAT TTAAATTGCA TCCAAAATAA AAATTGGATT ATGATATGAG TACAGAGTCG 2745
gggtc c tagac -- ----- a tgc g a c taat 225

GGAAGGGTAA TTTTGCATTG GATTTAAGTA TTCCAATTTT GAAAATATGA GTAAAGGATC 2685
t----- -- a / - c -- -- -- / 274
g gatca t gaataa

TATGGATGAA GATACAAAAA AGTTGATTTC CAATCGTAAC TAAACTTCT TTTGTTAAAG 2625
c g -- - t aa c tttgtaaagaa 340

GATAAGGAAG CCAATAGCT AAAAAACGAT AGTTTTGGTT TACTAGAACC ATCCGCATAT 2565
a tt a tt gac ga aa 400

TGTTTCAGCT CGGTGGAAC CCAATTCCTT TCCTCAGGAT CTCTTGAATG AAATTAGGGA 2505
t aa cc cta t at g aata ag 460

ACGAAGTAAG TAGATTAGAT GGATTTAGAT AGAATTCTTA TCTCCTACTC TATAAGGATC 2445
acg aat a ctagaa gg t t----- - c cc ----- g 508

ATCTAGAAAG CAGAGAGCTT TGGTTCATT CAAATAGAAA AGCTGACATA GATGTTAAGT 2385
c t attcgttt tatctgtatt cagacc- t g 567

gtagaattct tttttttttt cgaattttgt tcacatctta gatctataaa 617

GGTGAGAATA TCCATAAAGG AGCCGAATGA AATCAAAATT TCATGTTTCGG TTTTGAATTA 2325
ttgactcatc c g 677

=====3' exon=====
GAGACGTAA AAATAATCAA CCAACGTCGA CTATAACCCC TAGCCTTCCA AGCTAACGAT 2265
g t gt 737

=====
GCGGGTTCGA TTCCCGCTAC CCGCTCCATT CTGTATAAAA GGAGTATTCT ATTTGTCTAG 2205
t a tctat t tt ct aata tt taa a tc 797

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Figure 10. Homology between the barley (upper case letters) and tobacco (lower case) DNA sequence in the region of the intron-containing *trnG-UCC* gene. Blue = unaltered bases. Yellow = exon sequence. The “-35 box” and “-10 box” promoter sequences are indicated. Dashes (- -) represent deletions in the tobacco sequence. Insertions are represented by / . The sequence beneath / replaces the base above.

have arisen from duplication of the adjacent threonine tRNA gene with which it shares much sequence homology.

5.1.2. *trnG-GCC*

The non-coding strand of a second tRNA gene extends from 1558-1628. Judging by the anticodon sequence, GCC, it is a glycine tRNA gene. The predicted length of the mature RNA is 74 bases. The *Euglena gracilis* genes *trnG-GCC* and *trnG-UCC* both differ in 28 places (12, 14). The two *Euglena* genes form parts of tRNA gene-clusters, so it is not surprising to find no significant homology in the flanking regions with barley.

5.1.3. *trnS-UGA*

The non-coding strand of a third tRNA gene extends from 733-646. Judging by the anticodon sequence, UGA, the gene codes for a serine tRNA. The size of the mature tRNA is 91 nucleotides. The sequence of the gene is identical to the corresponding *trnS-UGA* from maize (21). There is a high homology in the flanking regions (see Figure 12). However, the sequence thought by KREBBERS et al. to be homologous to bacterial promoters is not totally conserved.

5.1.4. *trnFM*

The non-coding strand of a fourth tRNA gene extends from 2148 to 2075. Judging by its anticodon sequence, CAU, it is a methionine tRNA gene. The mature tRNA would be 77 nucleotides in length. The tRNA is predicted to be an initiator tRNA because of its homology to the *Phaseolus vulgaris* (5), spinach (4) and *Euglena* (14) initiator tRNAs. The putative barley tRNA-fmet differs from the spinach tRNA at 8 places, from the *Phaseolus* tRNA at 10 places and from the *Euglena* tRNA at 16 places. In contrast, the sequence of elongator methio-

nine tRNAs from *Euglena*, spinach and barley are different in 29, 41 and 43 positions, respectively (12, 28, 46). Thus, the barley gene described herein shows significantly greater homology to initiator than to elongator tRNA-mets.

The barley sequence shares with the *Phaseolus*, spinach and *Euglena* initiator tRNAs two characteristics of prokaryotic initiator tRNAs: the first nucleotide cannot base-pair and the sequence TTC is present in the T Ψ C loop, which in eukaryotic initiator tRNA generally has the sequence ATC instead. However, the A57 following the TTC, which is normally found in prokaryotic initiator tRNAs, is replaced by a G as in many eukaryotes. Thus, the barley fmet tRNA shows both prokaryotic and eukaryotic features.

The *Euglena* gene is part of a tRNA cluster. There is therefore no likelihood of homology with barley in the flanking regions. The structure of the tobacco gene has been reported in abstract form (40).

5.1.5. *trnG-UCC*

The sequence 5'GCGGGTTCGATTCCCGC was found at positions 2264-2248. Although the sequence contains the conserved features of a T Ψ C arm, a complete tRNA sequence could not be deduced from the flanking regions. A search was made for a sequence complementary to the 3' half of the putative amino-acyl stem, 2247-TACCCGC-2241, and was found at 2988-2982. Starting with this aminoacyl stem and the T Ψ C arm, a tRNA sequence with an intron in the D-stem was deduced. The length of the mature RNA is predicted to be 74 bases (2988-2966, 2287-2240). Judging by the anticodon sequence, UCC, it is a glycine tRNA. DENO and SUGIURA (9) have recently described a similar gene in tobacco plastid DNA. The sequence of the mature tRNAs are identical. They differ in 22 places from the *trnG-GCC* gene

[illegible]

Figure 11. Five examples of tandem or inverted repeated sequences. The symbol II indicates identity between the sequences.

described above and in 21 places from a *Euglena* trnG-UCC (12).

The most unusual feature of this tRNA gene is the presence of an intron. Introns have been found in genes for 5 chloroplast tRNAs: alanine, isoleucine, leucine, valine as well as glycine (8, 10, 17, 21, 39, 41, 46). The introns of the first four are in the anticodon loop, 3' to the anticodon. In contrast, the trnG genes from barley and tobacco both have the intron between bases 24 and 25 in the D-stem. The tobacco intron is 691 bp and is aligned for maximum homology with the 677 bp barley intron in Figure 10. The introns are identical for the first 17 and the last 13 bases. There are only 6 changes in the last 87 bases of the introns. There are also long identical stretches in the middle of the intron. The sequence CCA is found immediately downstream of the predicted end of the 3' exon. Since only one of these bases is conserved in tobacco it is likely that the 3' CCA of the mature tRNA is added posttranscriptionally as in all other plastid tRNAs. An ORF in the complementary strand in tobacco is not, however, conserved. The likely significance of this ORF is thus diminished.

In a study of intron/exon boundary sequences in the *Euglena rbcL* gene, KOLLER et al. (18) proposed consensus rules for these junctions. The 5' junction consensus is G₁G₂G and the 3' consensus is TTAPuTTTTAT. The glycine intron described here obeys the 5' consensus (GTGTG) but not the consensus at the 3' junction (CGTCGACTATAAC). The tobacco and barley valine 5' junctions (CGCGC) may also fit the consensus but the exact splice site is unknown. The seven other tRNA intron junctions do not fit the consensus. MICHEL and DUJON have studied the potential secondary structures of the introns of genes from lower eukaryotes, mitochondria and chloroplasts (25). They classified the introns into two classes on the basis of shared features. Two stem and loop structures near the 3' end of the intron are similar to structures found in group 2 introns (Figure 9b). It is interesting to note that two of the bases of this sequence which are altered in tobacco cause the replacement of a TA base pair with a GC base pair. Thus, substitutions in the primary structure have conserved the putative secondary structure.

5.1.6. Expression of the tRNA genes

Are the five tRNA genes expressed? This question has not been directly addressed. This would require the demonstration that the mature tRNAs hybridise specifically to the genes. Hybridisation of individually identified tRNA species to plastid DNA has been performed in a number of plants (43). For comparison with barley, the most relevant species is maize (37).

The physical maps of maize and barley plastid DNA are essentially colinear (29), so gene locations are likely to be identical. Two serine isoacceptors were found to hybridise to a *Sall*/*Bam*HI fragment corresponding to the barley fragment extending from 1499 to 4.6 kb nearer the proximal inverted repeat. A threonine tRNA was found to hybridise to a *Sall*/*Bam*HI fragment corresponding to the barley fragment extending from 2294 to 4.4 kb away from the proximal inverted repeat. As the serine tRNA gene was found at 733-646 and the threonine gene at 4112-4183 there is good evidence that these tRNA genes are expressed.

In contrast, initiator methionine and glycine tRNAs did not hybridise to the position in maize. This should not, however, be taken as definitive evidence that the two glycine and the methionine tRNA genes are not expressed. The technical problems associated with the purification and identification of tRNAs are such that no isoacceptor for glutamate, for example, has been mapped using these techniques (8).

As will be detailed below, it is clear that four of the tRNA genes are colinear with large RNA species. The exception is the serine tRNA gene. Establishing that these large RNAs are definitely processed into active tRNAs will require further study. The failure to detect a transcript which may be a precursor of the serine tRNA may be because the transcript is either rare or small (< 300 bp). Small molecules are likely to be lost during purification of the RNA or during transfer to Genescreen.

5.2. Repeated sequences

Heterogeneity between chloroplast genomes of different higher plants exists on two levels. Chloroplast genomes of plants from different families differ as a result of large (up to 50kbp)

inversions and deletions (27). Within genera the genomes' structures are very similar, differences being limited to small (up to 2 kbp) inversions and deletions (3, 11, 42).

The chloroplast genomes of the grasses (barley, wheat, maize) may be regarded as the ancestral higher-plant plastid genome (27). Their closest neighbours (tobacco, spinach) differ as a result of inversion of a ca. 22 kbp fragment extending in barley from about coordinate 70 to 92 (Figure 2). This latter site is within the fragment studied. Detailed restriction patterns have been determined for several members of the three genera, *Triticum* (3), *Oenothera* (11) and *Nicotiana* (42). Differences between members of the *Oenothera* spp. are placed randomly around the genome. In contrast, the differences between wheat spp. and between tobacco spp. are both localised in discrete regions. In both cases the region homologous to the region studied here in barley was frequently altered. TASSOPULU and KUNG (42) refer to this area as a recombination hot-spot.

Thus, both the small-scale differences within genera and the large-scale inversion distinguishing the grasses from tobacco and spinach involve the region studied in barley. Therefore, a search was made for repeated sequences which might have resulted in or from recombinations. The five largest repeats are shown in Figure 11 (see also Figure 1). Repeat A has two tandem members with 57 of the 62 bases conserved (allowing two inserts to maximise homology) and one inverted member with 20 bases conserved. The 3' ends of three trn genes (*fmet*, G-UCC and G-GCC) make up part of the sequences. The homology may suggest that the tRNAs evolved after gene-duplication events. Four other repeats (21 out of 25, 18 out of 22, 19 out of 20 and all 15 bases conserved) were noted. Two are inverted and two are tandem. As it may be calculated that a 15bp repeat would be expected every 10^9 bases, the frequency with which these sequences have been found in a 4294 bp sequence suggests a fundamental role. The presence of the long tandem repeats opens up the possibility of inserting foreign DNA at these sites by homologous recombination when transformation of the chloroplast is achieved.

AAGCTTCTCA AGCTCAAGCA TTTACTTTTC TAGTTAGAGA CCAGCGTCTT GGAGCTAATG	Barley
A S Q A Q A F T F L V R D Q R L G A N	Amino acid
	Maize
	Maize amino acid
TGGGATCCGC TCAAGGACCC ACAGGTTTTC GGAAATATCT AATCCGTTCC CCAACTGGGG	120
V G S A Q G P T G L G K Y L I R S P T G	
a g t c g g t g a	
	M
AGGTTATCTT TGGAGGGGAA ACTATGCGTT TTTGGGACCT TCGTGCTCCA TGGTTAGAAC	180
E V I F G G E T M R F W D L R A P W L E	
t g	
CTCTAAGGGG CCCCAACGGT TTGGACTTGA GTAGGTTGAA AAAAGACATA CAACCTTGGC	240
P L R G P N G L D L S R L K K D I Q P W	
c c	
	P
AAGAACGGCG CTCAGCAGAA TATATGACCC ACGCTCCTTT AGGCTCTTTA AATTCCGTGG	300
Q E R R S A E Y M T H A P L G S L N S V	
a t t	
GTGGCGTAGC GACCGAGATC AATGCAGTTA ATTATGTCTC TCCTAGAAGT TGGTTATCAA	360
G G V A T E I N A V N Y V S P R S W L S	
t c g g	
	A
CTTCTCATTT TGTCTAGGA TTCTTCTTTT TTGTGGGCCA TTTATGGCAT GCAGGAAGAG	420
T S H F V L G F F F F V G H L W H A G R	
c c	
CCCAGCTGC TGCAGCAGGT TTTGAAAAGG GAATCGATCG TGATTGGAA CCTGTTCTTT	480
A R A A A A G F E K G I D R D L E P V L	
t c	
ACATGAACCC TCTTAACATA GATTTTCTTA TTTATACCTG TTCTACTTTG TTTCTGTTCT	540
Y M N P L N +	
c - g t	
	T
GGCTCGGTTA TTCTATCTAG CCGAGCCATT CATTCCTTTT TATGAAAGAA AGATAAGGGA	600
c g t t	
	g g g g c t g c a c
CAGAATAAAA AAAAATGAAA GAAACAAACG TATTCAATAA GCAAAAGGAG AGAGAGGGAT	
a a c t g t a g t t a	
	TCCTC TCTCTCCCTA
	trNA ^{ser}

TCGAACCTC GATAGTTCCT AGAACTATAC CGGTTTTCAA GACCGGAGCT ATCAACCACT 720
 AGCTTGGGAG CTATCAAGGA TCTTGATATG GCCAAAAGTT CTGGCCTCGA TAGTTGGTGA
 CAGCCATCTC TCCACCGCCT AATCCTTATT TTACTCCTAC AAATAGAACA TAGCCATATA 780
 GTCGGTAGAG AGG
 AAAAAATAAA ATATTTATTA ACCTCTAGAA AGATATCAGA TACAAGTTCC TTTTCGATAT 840
 tgatct actaactcat c----- g c a c a
 ATCTCTGTAT ACTGTATACA CGGATACAGG ATCCGCTATA CCCGCTGTG AAATAAAGAC 900
 TAAATAACCT CCTCTCACCC CCATATCCAA ATAAAAAGA GGTTAAGTAA TAAATTTTAA 960
 c g a g
 TTAAAGAGAA GAATCAATGG ATTCATGATT AAACCCCTCC TACTTCTTGT ATTTTTTTAC 1020
 - a c ca
 AATTTTGGTT AAGTGAGGGA TCAAATATGT AGTCAACTTT ATTTGATGGT AGCTTGGAGG 1080
 a / a t
 caaatag
 ATTAGAAATA TGACTATTGC TTTCCAATTA GCTGTTTTTG CATTAAATGC GACTTCCTCA 1140
 M T I A F Q L A V F A L I A T S S
 t a
 N
 GTCTTAGTAA TTAGTGTAAC CCTTGATTTT GCTTCTCCTG ATGGTTGGTC AAATAATAAA 1200
 V L V I S V P L V F A S P D G W S N N K
 AATGTGTAT TTTCCGGTAC ATCATTATGG ATTGGACTAG TCTTCTCGT AGCTATTCTG 1260
 N V V F S G T S L W I G L V F L V A I L
 AATCTCTCA TTTCTTAAAT TAAATTTGGT TAATATTTAG TAACCCATA AAAAGAAAT 1320
 N S L I S +
 g -tg a - ----ta g c t taaa
 AATAAGGCC ATTTAAAAA TTCGAATAGT GAGACGTATT AAAACGCAAT TTGCGTTCGG 1380
 g /t cg a g tt tt ac t t t
 ctttt

Figure 13. Stem-loop structures close to the putative 3' end of transcripts. a) constitutive transcript family, b) the 0.3 kb light-induced transcript.

5.3. Open-reading frames

An open-reading frame which starts before the HindIII site (coordinate 1) extends for 500 bases into the sequenced fragment. It comprises 165 codons. A second, complete ORF extends from 1090 to 1278 and comprises 62 codons. The Shine-Dalgarno (36, 38) sequence GGAGG (1076-1080) precedes the start codon. No other ORFs of significance were found.

There is no homology between the deduced amino-acid sequences of these two ORFs and known primary sequences of chloroplast proteins. It is entirely possible that the ORFs are expressed as functional proteins because in an overlapping sequence from maize (19, 21) these ORFs are conserved. 1.3 kbp of a 3 kbp sequence from maize is homologous to the start of the barley sequence. Figure 12 shows the two DNA sequences aligned for maximum homology, together with the deduced amino-acid sequences. It can be seen that, despite 26 differences in the first 500 bases, there are only four amino acid changes. In the 62-codon ORF, only one nucleotide and one codon are altered. These five amino acid alterations are conservative substitutions. The Shine-Dalgarno sequence is conserved. This homology in the deduced amino acid sequences as well as the translational signal implies that a strict functional constraint on base substitutions exists and indicates that the ORFs are expressed as proteins.

In maize the long ORF was found to be 473 codons in length (Figure 1). Curiously the start

of this ORF is overlapped by a 353-codon ORF. The sequence of the 353-codon ORF is highly homologous to a pea chloroplast gene for the "D2" thylakoid protein (33). Both the 473- and 62-codon ORFs would code for hydrophobic and thus membrane proteins. At the present time there is no further information on the nature of the protein presumably encoded on these genes.

The rest of the maize and barley sequences which overlaps can be compared (Figure 12). Between the two ORFs but in the opposite direction is the tRNA gene for serine (21). The gene is identical in the two species but the immediate flanking regions are only moderately conserved. There are, however, areas of high or complete homology in the apparently non-coding sequences. For example there are only 6 changes in the 90 bases 501-590 and 4 changes in the 70 bases 971-1040. Differences between the sequences are normally caused by deletions or insertions (e.g. 785-815, 851-935). In two cases the ends of the deletions coincide with stretches of A residues (779-786, 933-939). The high level of sequence homology in much of this "non-coding" region again suggests that there is a functional constraint on base substitutions. It will take an experimental rather than a comparative approach to discover the reason for the constraint.

5.4. Transcription of pHvC35

5.4.1. The constitutive transcripts

Nine transcripts were found to hybridise to the end of the fragment nearer to the inverted repeat. Northern and S1-mapping studies (Figures 5 and 6) indicated that 4 of the transcripts ended beyond 350 and the remaining 5 at around 1812. The primary transcript is therefore processed from both the 3' and 5' ends. The role of this family of transcripts may be to produce mRNAs for the translation of the D2 protein and the proteins encoded by the 473- and 62-codon ORFs. They would also be precursors for the tRNA^{Arg}-GCC. A stem and loop structure frequently found at the end of chloroplast transcripts (45), can be formed from 1760-1780 (Figure 13a).

Although the 3' end of the 1.8 kb transcript

Figure 14. Scheme for the transcription and processing of the *trnG*-UUC and *trnM* loci. Boxes represent coding sequence. Named transcripts refer to the primary 1.14 kb transcript and the 0.86, 0.62 and 0.3 kb transcripts identified by Northern hybridisation.

was not determined precisely, its size and position indicate that it could be the mature mRNA for the 473-codon ORF. An mRNA suitable for the 62-codon ORF was not detected. It may have been too small and thus lost during purification of the RNA or during transfer to Genescreen. The lack of promoter sequences prior to the small ORF argues against it being separately transcribed.

The finding of a tRNA gene at the end of a large polycistronic transcript is similar to some transcripts of mammalian mitochondrial genomes (6). The processing of the 5.5 kb transcript into three mRNAs and into a precursor for a tRNA implies that the primary transcript is processed intercistronically. Multiple and/or polycistronic mRNAs have been found to hybridise to a number of chloroplast genes (7, 20, 26, 30). It has been proposed that a transcript encompassing the genes for the P 680 apoprotein, cytochrome b_6 and subunit IV of the cytochromes b_6/f complex is intercistronically processed (26). Many areas of the barley genome were found to hybridise to multiple or large (> 3

kb) transcripts (29). Thus, intercistronic processing of polycistronic transcripts is likely to be a general mechanism for the expression of plastid genes.

5.4.2. The 0.86, 0.62 and 0.3 kb light-induced transcripts

This family of transcripts are putative precursors and intermediates in the expression of the *trnM* and *trnG*-UCC genes. The time scale and extents of light-induction are similar, suggesting that they are developmentally related. A scheme for the complex transcription of this region of the DNA has to account for the following ends of transcripts determined by S1-mapping and Northern analyses: 5' 2988, 2888, 2714, 2195 and 2150; 3' 2238, 2178, 2167, 2000 and about 1850. A scheme to account for the Northern and S1-mapping data as well as the positions of the genes is shown in Figure 14.

A prokaryotic-like promoter sequence TT-GACA - 17 bp - CATAAT was found at 3027-2999 (Figure 10). The major 5' transcript

start was found at 2988 (Figure 7) 11 bases downstream. As no promoter-like sequence was found prior to the other transcription starts, it is proposed that 2988 is the start of the primary transcript. Northern analysis placed the 3' end of the 0.3 kb transcript at about 1850. A putative stem and loop transcription terminator sequence is found at 1895-1861 (Figure 13b). It is therefore likely that the primary transcript is 1.14 kb in length and extends from 2988 to about 1850. Failure to detect this transcript by Northern hybridisation suggests that it does not significantly accumulate.

After transcription of the putative 1.14 kb precursor, the first event is evidently the cleavage of the primary transcript. This would create the 5' starts of the 0.3 kb transcript found at 2195 or 2150. This latter position is only 2 bases from the start of the *trnJ*M gene. Following this step a second cleavage creates the 3' end found by S1-nuclease digestion at 2000. This cleavage forms two molecules of ca. 150 bases. These molecules and any further processing intermediates would be too small to be detected by Northern hybridisation as performed here. Further processing of both ends of the transcript as well as modified base formation would create the tRNA-fMet.

The other product from the initial cleavage of the putative 1.14 kb transcript is the extremely abundant 0.86 kb transcript (Figure 6). The 5' end of this molecule is at or close to the 5' base of the first exon of the intron-containing glycine tRNA gene, 2988. 3' ends for this molecule were found at both 2167 and 2178. Thus, the size of the molecule as determined by S1-mapping (810 and 821 b) is close to the size estimated by Northern analysis. Further processing would create the 3' end at 2238 which is close to the predicted 3' end of the 3' exon of the *trnG-UCC* gene.

Excision of the intron from the 0.86 kb transcript would create a 677 b molecule running from 2964-2288. A 5' end was located by S1-mapping at 2888. The 3' end of the 0.62 kb transcript was deduced by Northern analysis to be proximal to 2294. It is likely therefore that after excision of the intron, the bases between the 5' intron junction (2964) and the determined 5' end (2888) are rapidly removed.

If the 3' end of the transcript is similarly trimmed to about 2300, a molecule of 588 b is created. This is close to the size estimated by Northern analysis (0.62 kb). Further processing of the metastable 0.62 kb molecule would create the 5' start observed at 2714. The role of the intron-containing transcripts is unknown.

After removal of the intron, the 0.86 kb transcript would be reduced to about 240 b. Further processing of this molecule would create the *trnG-UCC* gene product. It is clear that further experiments with smaller S1 probes and capping enzyme are required to substantiate this hypothesis.

5.4.3. The 0.8 and 1.8 kb light-induced transcripts

A 5' end corresponding to either or both of the 0.8 and 1.8 kb light-induced transcripts was found at 3073 ± 1 . This is 6-8 bases downstream from the sequence TTGACA - 18 bp - TATTGA (3039-3067) similar to the consensus of bacterial and chloroplast promoters (8). The 0.8 kb transcript would extend to about 3900, whereas the 1.8 kb transcript would extend beyond the end of the sequence. The longer transcript could act as a precursor for the tRNA^{thr} found at 4112-4183. The putative end of the shorter transcript falls in a sequence which is both AT-rich and capable of forming large secondary structures (Figure 9a). These features may act as transcription terminators, attenuators or as processing sites. No significant ORFs or other tRNA genes were found in the sequence between 3073 and 4112. It is therefore difficult to suggest a role for the shorter transcript at present.

5.4.4. The 0.72 kb light-induced transcript

Northern analysis indicates that the 3' end of this transcript falls between 3632 and 3795. This implies that both strands of the DNA in this region are transcribed. Analysis of the sequence of the adjacent chloroplast DNA fragment will be required to elucidate the role of this transcript.

5.5. Summary

The earlier study had indicated that this area of DNA was disproportionately heavily transcribed (29). This has been amply confirmed. Fifteen transcripts totalling 38 kb have been identified as hybridising under stringent conditions to this 4.3 kbp fragment. Roles have been suggested for 14 of the transcripts. Transcripts hybridise to all but about 140 bases of the DNA. In one area transcripts hybridise to both strands. The promoter sequences (TTGACA etc.) of the 0.86 and 1.8 kb light-induced transcripts are separated by only 10 bases. It remains to be seen whether the entire chloroplast genome is transcribed as completely as this fragment.

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REFERENCES

1. BERK, A.S. & P.A. SHARP: Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell* 12, 721-732 (1977)
2. BIRNBOIM, H.C. & J. DOLY: A rapid alkaline extraction procedure for screening recombinant DNA. *Nucleic Acids Res.* 7, 1513-1523 (1979)
3. BOWMAN, C.M. & T.A. DYER: Structural variation and the occurrence of small repeated sequences in the chloroplast genome of wheat and its close relatives. Abstract Nato Advanced Studies Institute FEBS Advanced Course: Molecular Form and Function of the Plant Genome. Renesse, The Netherlands, B2 (1984)
4. CALAGAN, J.L., R.M. PIRTLE, I.L. PIRTLE, M.A. KASHDAN, H.J. VREMAN & B.S. DUDOCK: Homology between chloroplast and prokaryote initiator tRNA. Nucleotide sequence of spinach chloroplast methionine initiator tRNA. *J. Biol. Chem.* 255, 9981-9984 (1980)
5. CANADAY, J., P. GUILLEMAUT & J.H. WEIL: The nucleotide sequences of the initiator transfer RNAs from bean cytoplasm and chloroplasts. *Nucleic Acids Res.* 8, 999-1008 (1980)
6. CLAYTON, D.A.: Transcription of the mammalian mitochondrial genome. *Ann. Rev. Biochem.* 53, 573-594 (1984)
7. CROSSLAND, L.D., S.R. RODERMEL & L. BOGORAD: Single gene for the large subunit of ribulosebisphosphate carboxylase in maize yields two differentially regulated mRNAs. *Proc. Natl. Acad. Sci.* 81, 4060-4064 (1984)
8. CROUSE, E.J., H.J. BOHNERT & J.M. SCHMITT: Chloroplast RNA synthesis. In: *Chloroplast Biogenesis*, R.J. Ellis ed., Seminar Series, Soc. Exp. Biol., Cambridge University Press, Vol. 21, 83-136 (1984)
9. DENO, H. & M. SUGIURA: Chloroplast tRNA^{Gly} gene contains a long intron in the D stem: nucleotide sequences of tobacco chloroplast genes for tRNA^{Gly} (UCC) and tRNA^{Arg} (UCU). *Proc. Natl. Acad. Sci.* 81, 405-408 (1984)
10. DENO, H., A. KATO, K. SHINOZAKI & M. SUGIURA: Nucleotide sequences of tobacco chloroplast genes for elongator tRNA^{Met} and tRNA^{Val} (UAC): the tRNA^{Val} (UAC) gene contains a long intron. *Nucleic Acids Res.* 10, 7511-7520 (1982)
11. GORDON, K.H.J., E.J. CROUSE, H.J. BOHNERT & R.G. HERRMANN: Physical mapping of differences in chloroplast DNA of five wild-type plastomes in *Oenothera* subsection *Euoenothera*. *Theor. Appl. Genetics* 61, 373-384 (1982)
12. HOLLINGSWORTH, M.J. & R.B. HALLICK: *Euglena gracilis* chloroplast transfer RNA transcription units. Nucleotide sequence analysis of a tRNA^{Tyr}-tRNA^{His}-tRNA^{Met}-tRNA^{Trp}-tRNA^{Glu}-tRNA^{Gly} gene cluster. *J. Biol. Chem.* 257, 12795-12799 (1982)
13. HU, N.-T. & J. MESSING: The making of strand-specific M13 probes. *Gene* 17, 271-277 (1982)
14. KARABIN, G.D. & R.B. HALLICK: *Euglena gracilis* chloroplast transfer RNA transcription units. Nucleotide sequence analysis of a tRNA^{Thr}-tRNA^{Gly}-tRNA^{Met}-tRNA^{Ser}-tRNA^{Gln} gene cluster. *J. Biol. Chem.* 258, 5512-5518 (1983)
15. KASHDAN, M.A. & B.S. DUDOCK: Structure of a spinach chloroplast threonine tRNA gene. *J. Biol. Chem.* 257, 1114-1116 (1982)
16. KASHDAN, M.A., R.M. PIRTLE, I.L. PIRTLE, J.L. CALAGAN, H.-J. VREMAN & B.S. DUDOCK: Nucleotide sequence of a spinach chloroplast threonine tRNA. *J. Biol. Chem.* 255, 8831-8835 (1980)
17. KOCH, W., K. EDWARDS & H. KÖSSEL: Sequencing

- of the 16S-23S spacer in a ribosomal RNA operon of *Zea mays* chloroplast DNA reveals two split tRNA genes. *Cell* 25, 203-213 (1981)
18. KOLLER, B., J.C. GINGRICH, G.L. STIEGLER, M.A. FARLEY, H. DELIUS & R.B. HALLICK: Nine introns with conserved boundary sequences in the *Euglena gracilis* chloroplast ribulose-1,5-bisphosphate carboxylase Gene. *Cell* 36, 545-553 (1984)
 19. KREBBERS, E.T.: Ph. D. Thesis. Harvard University (1983)
 20. KREBBERS, E.T., I.M. LARRINUA, L. MCINTOSH & L. BOGORAD: The maize chloroplast genes for the β and ϵ subunits of the photosynthetic coupling factor CF₁ are fused. *Nucleic Acids Res.* 10, 4985-5002 (1982)
 21. KREBBERS, E., A. STEINMETZ & L. BOGORAD: DNA sequences for the *Zea mays* tRNA genes tV-UAC and tS-UGA: tV-UAC contains a large intron. *Plant Mol. Biol.* 3, 13-20 (1984)
 22. KUNTZ, M., J.H. WEIL & A. STEINMETZ: Nucleotide sequence of a 2 kbp BamHI fragment of *Vicia faba* chloroplast DNA containing the genes for threonine, glutamic acid and tyrosine transfer RNAs. *Nucleic Acids Res.* 12, 5037-5041 (1984)
 23. MAXAM, A.M. & W. GILBERT: Sequencing end-labelled DNA with base-specific chemical cleavages. *Meth. Enzymol.* 65, 499-560 (1980)
 24. MESSING, J. & J. VIEIRA: A new pair of M13 vectors for selecting either DNA strand of double digested restriction fragments. *Gene* 19, 269-276 (1982)
 25. MICHEL, F. & B. DUJON: Conservation of RNA secondary structures in two intron families including mitochondrial-, chloroplast-, and nuclear-encoded members. *EMBO J.* 2, 33-38 (1983)
 26. MORRIS, J. & R.G. HERRMANN: Nucleotide sequence of the gene for the P680 chlorophyll a apoprotein of the photosystem II reaction center from spinach. *Nucleic Acids Res.* 12, 2837-2850 (1984)
 27. PALMER, J.D. & W.F. THOMPSON: Chloroplast DNA rearrangements are more frequent when a large inverted repeat sequence is lost. *Cell* 29, 537-550 (1982)
 28. PIRTLE, R.M., J.L. CALAGAN, I.L. PIRTLE, M.A. KASHDAN, H.S. VREMAN & B.S. DUDOCK: The nucleotide sequence of spinach chloroplast methionine elongator tRNA. *Nucleic Acids Res.* 9, 183-188 (1981)
 29. POULSEN, C.: The barley chloroplast genome: Physical structure and transcriptional activity in vivo. *Carlsberg Res. Commun.* 48, 57-80 (1983)
 30. POULSEN, C.: Two mRNA species differing by 258 nucleotides at the 5' end are formed from the barley chloroplast rbcL gene. *Carlsberg Res. Commun.* 49, 89-104 (1984)
 31. OLIVER, R.P., C.G. KANNANGARA & S.P. GOUGH: Structure and functions of a highly transcribed fragment of barley chloroplast DNA. An RNA involved in Δ -aminolaevulinic acid biosynthesis. Abstract presented at the NATO Advanced Studies Institute. FEBS Advanced Course: Molecular Form and Function of the Plant Genome. Renesse, The Netherlands, B22 (1984)
 32. OLIVER, R.P. & C. POULSEN: The barley chloroplast genome: Analysis of a highly transcribed region. *Proc. VI Int. Congr. Photosynthesis*, Brussels, Belgium. ed. C. Sybesma, Martinus Nijhoff/W. Junk. Publ. vol. IV part 5 pp. 521-524 (1984)
 33. RASMUSSEN, O.F., G. BOOKJANS, B.M. STUMMANN & K.W. HENNINGSEN: Localisation and nucleotide sequence of the gene for the membrane polypeptide D2 from pea chloroplast DNA. *Plant Mol. Biol.* 3, 191-199 (1984)
 34. RASMUSSEN, S.K., H.E. HOPP & A. BRANDT: Nucleotide sequences of DNA clones for B1 hordein polypeptides. *Carlsberg Res. Commun.* 48, 187-199 (1983)
 35. SANGER, F., S. NICKLEN & A.R. COULSON: DNA sequencing unit chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* 74, 5463-5467 (1977)
 36. SCHWARZ, Z. & H. KOSSEL: The primary structure of 16S rDNA from *Zea mays* chloroplast is homologous to *E. coli* 16S rDNA. *Nature* 283, 739-742 (1980)
 37. SELDEN, R.F., A. STEINMETZ, L. MCINTOSH, L. BOGORAD, G. BURKARD, M. MUBUMBILA, M. KUNTZ, E.J. CROUSE & J.H. WEIL: Transfer RNA genes of *Zea mays* chloroplast DNA. *Plant Molec. Biol.* 2, 141-153 (1983)
 38. SHINE, J. & L. DALGARNO: Determinant of cistron specificity in bacterial ribosomes. *Nature* 254, 34-38 (1975)
 39. STEINMETZ, A., E.J. GUBBINS & L. BOGORAD: The anticodon of the maize chloroplast gene for tRNA^{Leu}_{UAA} is split by a large intron. *Nucleic Acids Res.* 10, 3027-3037 (1982)
 40. SUGIURA, M., K. SHINOZAKI, H. DENO, T. KAMOGASHIRA & M. SUGITA: Structure of tobacco chloroplast tRNA genes. *Int. tRNA Workshop. Abstract.* Hakone, Japan (1983)
 41. TAKAIWA, F. & M. SUGIURA: Nucleotide sequence of the 16S-23S spacer region in a rRNA gene cluster from tobacco chloroplast DNA. *Nucleic Acids Res.* 10, 2665-2676 (1982)
 42. TASSOPULU, D. & S.D. KUNG: *Nicotiana glauca* chloroplast genome 6. Deletion and hot spot - a proposed origin of the inverted repeats. *Theor. Appl. Genet.* 67, 185-193 (1984)
 43. WEIL, J.H., M. MUBUMBILA, M. KUNTZ, M. KELLER, E.J. CROUSE, G. BURKARD, P. GUILLEMAUT, R. SELDEN, L. MCINTOSH, L. BOGORAD, W.

- LÖFFELHARDT, H. MUCKE, H.J. BOHNERT, A. DIETRICH, G. SOUCIET, B. COLAS, P. IMBAULT & V. SARONTOGLOU: Comparative studies on tRNA and aminoacyl-tRNA synthetases from various photosynthetic organism. In: Structure and Function of Plant Genomes Eds. O. Ciferri & L. Dure. In: ASI Ser. A, Life Sciences vol 63. Plenum Press N.Y. pp. 167-180 (1983)
44. WETTSTEIN, D. VON, C. POULSEN, R.P. OLIVER, S.P. GOUGH, C.G. KANNANGARA, B.L. MØLLER & D. SIMPSON: Concerted interplay of chloroplast and nuclear genes in the formation of the photosynthetic apparatus. Proc. XV Int. Congr. Genetics. New Delhi, India 1984 (in press)
45. WHITFIELD, P.R. & W. BOTTOMLEY: Organisation and structure of chloroplast genes. Ann. Rev. Plant Physiol. 34, 279-310 (1983)
46. ZURAWSKI, G. & M.T. CLEGG: The barley chloroplast DNA *atpBE*, *trnM2* and *trnV1* loci. Nucleic Acids Res. 12, 2549-2559 (1984)

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