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Nucleotide sequence of a 2 kbp BamH I fragment of *Vicia faba* chloroplast DNA containing the genes for threonine, glutamic acid and tyrosine transfer RNAs

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ABSTRACT

The entire nucleotide sequence of a 2014 bp BamH I fragment from broad bean (Vicia faba) chloroplast DNA containing the genes for $tRNA^{Thr}$ (trnT), $tRNA^{Glu}$ (trnE) and $tRNA^{Tyr}$ (trnY) has been determined. The $tRNA^{Glu}$ and $tRNA^{Tyr}$ genes are separated by only 60 bp and are probably part of the same transcriptional unit. The $tRNA^{Thr}$ gene is located on the complementary strand, 876 bp away from the $tRNA^{Glu}$ gene. This fragment also contains an open reading frame of 82 codons, as well as a series of AT-rich, direct and inverted repeats.

INTRODUCTION

Whereas a number of chloroplast proteins are nuclear-encoded, synthesized in the cytoplasm and subsequently transported into the plastids, the chloroplast-encoded proteins are synthesized by a chloroplast-specific translation machinery. This protein synthesizing apparatus contains nuclear-encoded elements such as a number of ribosomal proteins (1), aminoacyl-tRNA synthetases (2, 3) and elongation factor EF Ts (4). At least 11 ribosomal proteins (5), the elongation factors EF Tu (6-8) and EF G (8), as well as the rRNAs and tRNAs (9), are plastid-encoded.

Chloroplast transfer RNA genes have been mapped on the plastid genome of Euglena (10) and of 6 different higher plant species, namely spinach, maize, common bean, tobacco, broad bean, wheat and pea (11-17), as well as on the cyanelle genome of Cyanophora paradoxa (18). In higher plant chloroplasts, most of these gene maps show similar patterns, although evidence for rearrangements can be found. The most striking differences in the general organization of genes are found in the case of the chloroplast genomes of some legumes, such as broad bean and pea, which are significantly shorter (about 30 kbp) than those of most higher plants. They have no large inverted repeated region and contain a single set of ribosomal RNA genes (19, 20). But even pea and broad bean differ significantly from one another in the general organization of their plastid genome (21).

The study of the organization and structure of the tRNA genes should provide extensive information on the evolution of plastid genomes. Physical maps of the 123 kbp broad bean plastid genome have been published (19, 22) and a number of chloroplast tRNA genes have been localized (15), but no sequence data on tRNA genes are available, except for the split $tRNA^{Leu}$ gene (23) which shows high sequence homology with that of maize (24).

We describe here the organisation and nucleotide sequence of three chloroplast tRNA genes from broad bean: a $tRNA^{Thr}$ gene, whose structure is very similar to that sequenced in the case of spinach chloroplasts (25), and the genes for $tRNA^{Glu}$ and $tRNA^{Tyr}$.

MATERIALS AND METHODS

Isolation of chloroplast DNA and tRNAs as well as cloning of chloroplast DNA fragments into pBR322 have been described (23). The clone containing fragment Bam 19 (about 2 kbp) was one of the clones that hybridized very strongly to labeled chloroplast tRNAs. It was designated pVfc 26. Recombinant DNA was isolated as already described (26).

Fragment Bam 19 was localized on the broad bean restriction map by hybridization of nick-translated (27) recombinant plasmid pVfc 26 to Southern blots (28) containing chloroplast DNA digested with restriction endonucleases Sal I and Kpn I.

The nucleotide sequence was determined by the dideoxy chain termination method (29) as well as the chemical cleavage method (30). For dideoxy-sequencing, subfragments of Bam 19 were cloned into suitable restriction sites of the replicative form of M13 derivatives mp8 and mp9. The chemical method was used for the determination of the nucleotide sequence from both ends of the unique Nco I site, as well as of a number of Hinf I fragments. The Nco I site was labeled using the large fragment of *E. coli* DNA polymerase (Klenow fragment) in the presence of $^{32}P\,\alpha$-dCTP and the labeled fragment was subsequently redigested with BamH I. The single end-labeled fragments were separated in a LMT Agarose gel (Seaplaque), isolated from the gel by phenol extraction of the melted gel (65° C) and precipitated with one volume of isopropanol in the presence of 10 µg/ml carrier tRNA. After centrifugation, the pellet was resuspended in 200 µl of 0.3 M Na acetate and precipitated with ethanol. After one ethanol wash (70% ethanol) the pellet was dried, redissolved in 100 µl distilled water and used for sequencing. Fragments labeled at the Hinf I site were first fractionated on a 6% polyacrylamide gel; the individual fragments were then

strand-separated before the chemical chain-breaking reactions were performed (30).

RESULTS AND DISCUSSION

Location of fragment Bam 19 on the broad bean chloroplast genome

The broad bean chloroplast DNA fragment present in the clone pVfc 26 was identified by agarose gel electrophoresis of the BamH I digested plasmid as Bam 19 which is about 2 kbp long. Since the physical map of the BamH I fragments has not yet been determined on the broad bean chloroplast genome, Bam 19 had to be located via hybridization of nick-translated Bam 19 to Southern blots carrying Sal I and Kpn I fragments for which the physical maps had been published (19). Bam 19 hybridizes exclusively to Sal fragment 1 and Kpn 4 (data not shown), suggesting that Bam 19 is entirely contained in these two fragments (no Kpn I and no Sal I sites could be found in the nucleotide sequence of Bam 19).

Mubumbila et al. (15) have shown that Kpn 4 (12.5 kbp) hybridizes to at least 6 tRNAs which include $tRNA^{Arg}_{1}$, $tRNA^{Asp}_{1}$, $tRNA^{Asp}_{2}$, $tRNA^{Thr}$, $tRNA^{Tyr}$ and $tRNA^{Pro}$. Since the clone pVfc 26 showed strong hybridization to radioactively labeled total chloroplast tRNAs, the genes of at least several of the above-mentioned tRNAs were therefore expected on fragment Bam 19.

DNA sequence analysis of fragment Bam 19

A detailed restriction map and the sequencing strategy of fragment Bam 19 is shown in Figure 1. EcoR I, Bgl II, Hpa II, Taq I and Sau 3A sub-fragments have been cloned into the replicative form of M13 mp8 and mp9

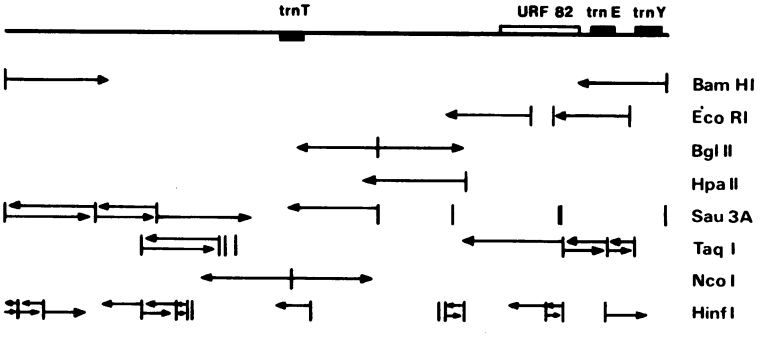

Figure 1 . Physical map of broad bean DNA fragment Bam 19 and sequencing strategy. tRNA genes are shown in black boxes, the unidentified reading frame (URF 82) in an open box. For details see text.

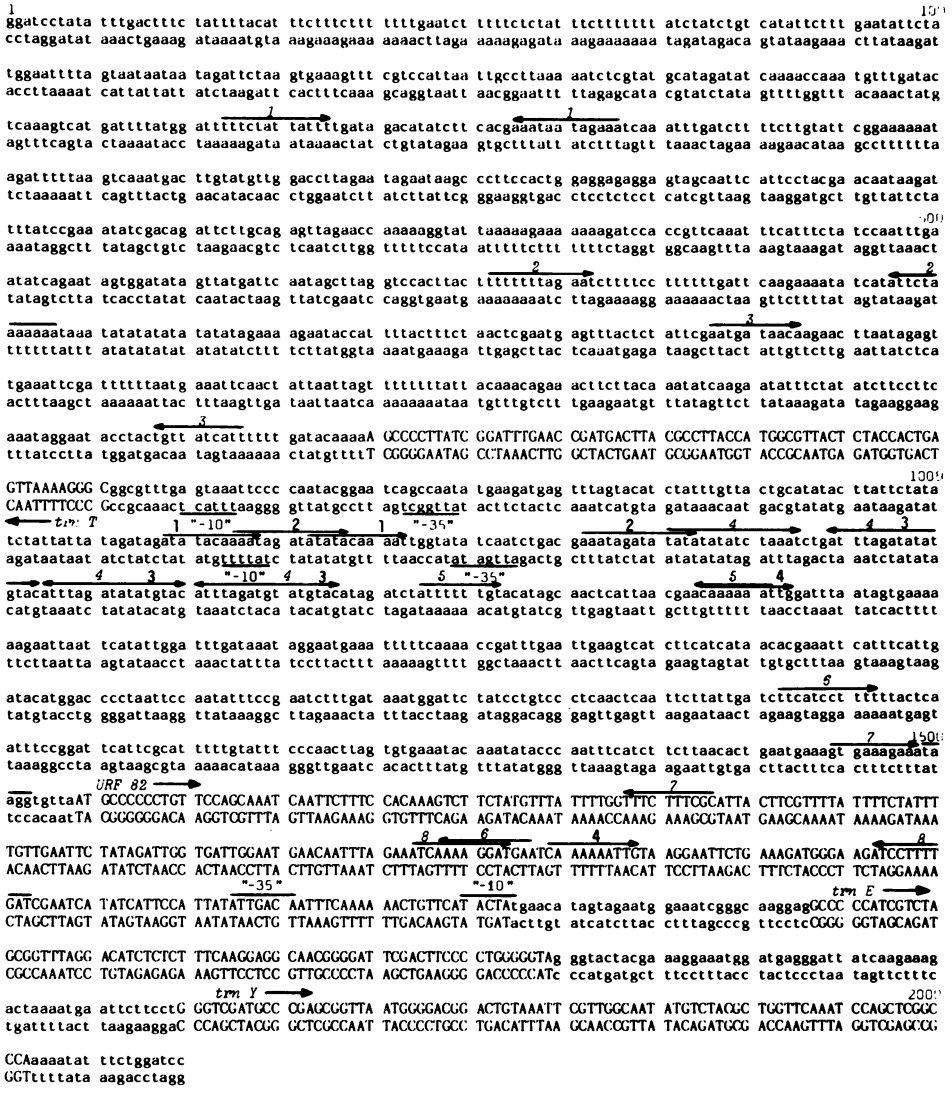

Figure 2 . Nucleotide sequence of fragment Bam 19. Intergenic regions are given in lower case letters, genes in upper case. Arrows indicate repeated regions: inverted repeats are indicated by numbers in italics (*1* to *8*), direct repeats (1 to 4) are indicated by bold numbers.

and sequenced according to Smith (29). The complete nucleotide sequence of Bam 19 is given in Figure 2. Three tRNA genes were identified by their anticodon sequence, as well as by comparison with tRNA sequences already known for other organisms. They are trnT (encoding $tRNA^{Thr}$), trnE (encoding

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1494  S.D.
AGAAATAAGGTGTTA ATG CCC CCC TGT TCC AGC AAA TCA ATT CTT TCC ACA AAG TCT TCT ATG
                Met Pro Pro Cys Ser Ser Lys Ser Ile Leu Ser Thr Lys Ser Ser Met

TTT ATT TTG GTT TCT TTC GCA TTA CTT CGT TTT ATT TTC TAT TTT GTT GAA TTC TAT AGA
Phe Ile Leu Val Ser Phe Ala Leu Leu Arg Phe Ile Phe Tyr Phe Val Glu Phe Tyr Arg

TTG GTG ATT GGA ATG AAC AAT TTA GAA ATC AAA AGG ATG AAT CAA AAA ATT GTA AGG AAT
Leu Val Ile Gly Met Asn Asn Leu Glu Ile Lys Arg Met Asn Glu Lys Ile Val Arg Asn

TCT GAA AGA TGG GAA GAT CCT TTT GAT CGA ATC ATA TCA TTC CAT TAT ATT GAC AAT TTC
Ser Glu Arg Trp Glu Asp Pro Phe Asp Arg Ile Ile Ser Phe His Tyr Ile Asp Asn Phe
                                          1770
AAA AAA CTG TTC ATA CTA TGA ACA TAG TAG AATG
Lys Lys Leu Phe Ile Leu  *  Thr  *   *
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Figure 3 . Nucleotide sequence (and corresponding amino acid sequence) of URF 82. S.D. (Shine-Dalgarno sequence) indicates a possible ribosome binding-site.

$tRNA^{Glu}$) and trnY (encoding $tRNA^{Tyr}$). Whereas the genes for $tRNA^{Thr}$ and $tRNA^{Tyr}$ had been mapped in this region by Mubumbila et al. (15), the finding of a $tRNA^{Glu}$ gene on this fragment was unexpected. In fact this gene had never been mapped on any of the higher plant chloroplast genomes which have been studied. The failure to map this gene was due to technical problems encountered during identification of the purified tRNAs (see ref. 11-17). The positions of the three genes on Bam 19 are the following: trnT starts at position 911 and extends to position 840 (direction of transcription); the complementary strand contains trnE which begins at position 1787 and ends at position 1859; trnE is followed, on the same DNA strand, by trnY which extends from position 1920 to position 2003.

An open reading frame with a coding capacity for an 82 amino acid-long polypeptide (URF 82) is found 5' to trnE (positions 1509 to 1754). It is preceded by a potential ribosome binding site (TAAGG) six nucleotides upstream. It is followed by 3 stop codons, the first being UGA, the other two (separated by ACA) being UAG. The protein sequence derived from the putative gene (Figure 3) shows no similarities with either known ribosomal proteins from E. coli (A. Subramanian, personal communication) or any other chloroplast proteins identified so far. It contains an exceptionally low amount of Ala and Gly (1.2% each) and is very rich in Phe (12.2%). Furthermore its amino end is very rich in hydroxy-amino acids (6 Ser and one Thr are found in the first 15 amino acids).

Interesting to point out is the fact that the 3' end of this putative protein gene contains the sequences ATTGACA ("-35-like region") and TACTAT ("-10-like region"), separated by 18 nucleotides, which could be signals for

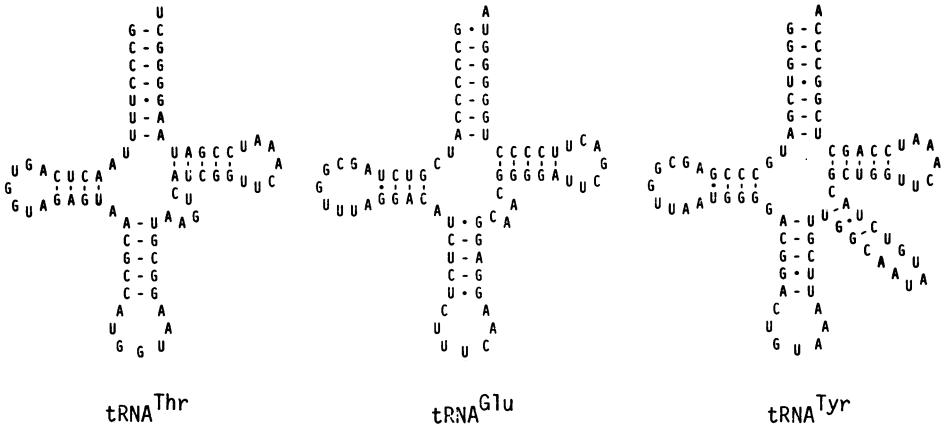

Figure 4 . Cloverleaf structures of broad bean chloroplast $tRNA^{Thr}$, $tRNA^{Glu}$ and $tRNA^{Tyr}$ deduced from the sequences of their genes: the 3'terminal CCA as well as modified nucleotides are not shown.

the transcription of trnE and trnY (see below).

Features of the three tRNAs

The cloverleaf structures of the three tRNAs (as deduced from the sequence of their genes) are shown in Figure 4. The $tRNA^{Thr}_{GGU}$ is 75 nucleotides long. It shows a single base difference with the spinach chloroplast $tRNA^{Thr}_{GGU}$ (25): it has a U in the 5th position, leading to a U - G base pair instead of a C - G. This tRNA has 77% homology with chloroplast $tRNAs^{Thr}_{UGU}$ from Euglena (31) and maize (32) and 64% homology with E. coli $tRNA^{Thr}_{GGU}$ (33).

The $tRNA^{Glu}_{UUC}$ is 76 nucleotides long. It has an unusual feature which is the presence of an A_{53} - U_{61} pair instead of the G_{53} - C_{61} pair usually found in the TΨ arm. Unusual base pairs at this position have been found in the $tRNA^{Glu}$ from Euglena chloroplasts (34) as well as in some fungal and mammalian mitochondrial tRNAs, as well as in some phage tRNAs (33). Its presence in chloroplast $tRNA^{Glu}_{UUC}$ from broad bean and Euglena suggests that this might be one distinctive chloroplast feature for this tRNA. The homology with the Euglena chloroplast isoacceptor is 92% and with that from E. coli 72%. The identification of this gene in higher plant chloroplasts is of particular interest since the $tRNA^{Glu}$ was the only higher plant chloroplast tRNA for which no information (either from mapping or from sequencing studies) was available to date.

The $tRNA^{Tyr}$ is 87 nucleotides long. Its anticodon is GUA. This is the

normal anticodon for tyrosine, different from the CUA anticodon found in suppressor $tRNA^{Tyr}$ in bacteria (33) and also in tobacco cytoplasm (35). Sequence homology is 82%, 76% and 68% with the isoacceptors from Scenedesmus chloroplasts (36), Euglena chloroplasts and E. coli , respectively.

Features of the three tRNA genes and their flanking regions

None of the three tRNA genes described here encodes CCA, the 3'terminal sequence of mature tRNAs. This seems to be a rule in chloroplast tRNA genes which has no exception so far. This feature, also found in nuclear tRNA genes, is optional in bacteria, where the large majority of tRNA genes, however, encode the CCA. Whether the strict observance of this rule in chloroplasts and nuclei has any significance is unknown, but it may be important for the maturation processes.

The study of the flanking regions of chloroplast tRNA genes and the comparison with those of the corresponding genes from other plant chloroplasts should provide information on the sequences involved in the control of the expression of these genes. Most of the chloroplast genes studied so far, tRNA as well as protein genes, have been shown to contain, in their 5'flanking region, two short stretches of sequences which resemble the "-35" and "-10" regions of prokaryotic promoters (32, 37). In several cases these sequences are embedded in a very highly conserved region which can extend up to 60 base pairs or more upstream of the gene (38). We can therefore assume that chloroplast promoters are present in the 5'flanking regions of the genes and possibly consist, at least in part, of the above-mentioned "-35" and "-10" regions.

Among the three tRNA genes described here, only that for $tRNA^{Glu}$ contains in its upstream region a promoter-like structure: a possible "-10" region (TACTAT) is found 32 nucleotides upstream of the nucleotide corresponding to the 5'end of the mature tRNA. This "-10" region is separated by 18 nucleotides from a possible "-35" region (ATTGACA). As mentioned above, these promoter-like sequences are located within the 3'end of the URF 82 open reading frame. Several possibilities therefore exist for the initiation of transcription of the $tRNA^{Glu}$ gene: it could be transcribed from the promoter of the protein gene, or it could be transcribed from its own promoter, or both.

The $tRNA^{Tyr}$ gene, located 60 bp downstream of the $tRNA^{Glu}$ gene, is probably co-transcribed with the $tRNA^{Glu}$ gene since no terminator-like structure for the $tRNA^{Glu}$ and no promoter-like sequence (except TAAAAT) for $tRNA^{Tyr}$ are found in the intergenic region.

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950                          "-10"          trn T ⟶
TATTGGCTGATTCCGTATTGGGGAATTTACTCAAACGCCGCCCTTTTAACTCAGTGGTAGAGTAACGCCA
 **   * **  *   *     ***** *** *** *  **** **************************
AAT   CAGA  C   A    AGAATTGACTAAAAGG  GCCCCTTTAACTCAGTGGTAGAGTAACGCCA
                                                                    817
TGGTAAGGCGTAAGTCATCGGTTCAAATCCGATAAGGGGCTTTTT  GTA  TCA AAAAA TGATAACA
*****************************************  **   *** ***** *     **
TGGTAAGGCGTAAGTCATCGGTTCAAATCCGATAAGGGGCTTTTGAGGTTTTTCATAAAAACTC    CA
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Figure 5 . Comparison of broad bean and spinach chloroplast trnT. Asterisks indicate identical nucleotides. Black dots mark first and last nucleotides of the deduced mature tRNAs.

The search for promoter-like regions upstream of the tRNA^{Thr} gene revealed two possible promoter regions: one in the immediate upstream region (920-949) with a "-10" region TTTACT and a "-35" region ATTGGCT; a second promoter-like structure, further upstream (1024-1055), with a "-10" region TATTTT and a "-35" region ATTGATA. Since the corresponding gene from spinach chloroplasts has been sequenced (25), it was interesting to compare the flanking regions of both the spinach and the broad bean genes. However the published sequence from the spinach gene includes only 25 nucleotides of the 5'flanking region. Little information can therefore be obtained from this comparison, but it should be mentioned that a short conserved region shows a possible "-10" region (TTGACT) similar to the TTTACT of the immediate upstream region of the broad bean gene (Figure 5).

The 3'flanking region of broad bean chloroplast trnT exhibits little sequence homology with the corresponding region of spinach chloroplast trnT, except immediately downstream, where a short stretch of "T"s, as well as a

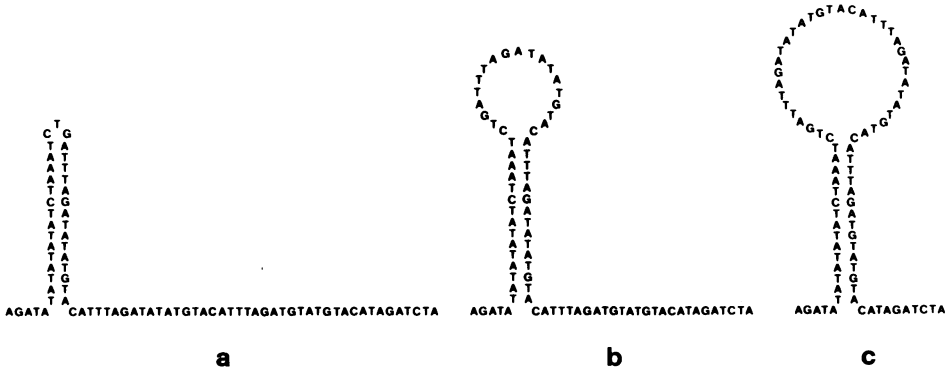

Figure 6 . Three possible secondary structures of the region between nucleotides 1071 to 1136. See text for details.

short stretch of "A"s, are conserved and might serve as a signal for termination of transcription. T-stretches immediately following the genes were found in some other chloroplast tRNA genes such as maize $tRNA^{Thr}_{UGU}$ gene (32) and Euglena $tRNA^{Ser}_{GCU}$ gene (31). In spinach chloroplasts, a short open reading frame overlapping the $tRNA^{Thr}$ gene was found in this region, but this is not the case in broad bean.

Fragment Bam 19 contains many direct and inverted repeated sequences

Fragment Bam 19 displays an unusually high frequence of direct and inverted repeat sequences. The most interesting set of repeats is found between trnT and trnE, beginning at position 1071 and extending to position 1136. The repeat unit comprises 16 nucleotides which are tandemly repeated three times without spacer nucleotides. It is very AT-rich (14 out of 16 nucleotides are AT). In the third unit a G replaces an A at position 9 of the repeat unit. More interesting even is the fact that each copy of this direct repeat can base-pair to a segment which has exactly the complementary sequence in opposite orientation (dyad sequence) preceding the first copy of the direct repeats. As a consequence, this could lead to a hairpin structure if the two closest regions base-pair (Figure 6a), or to a stem and loop structure with either one (Figure 6b) or two (Figure 6c) repeat units in the loop. This structure, because of its high AT content, could facilitate the separation of the DNA strands (for the initiation of chloroplast DNA replication, for instance).

Other direct and inverted repeats have been identified on fragment Bam 19 and some are shown in Figure 2. We have no idea of the significance of these repeated sequences, but some of them might have been involved in the rearrangement of the broad bean chloroplast genome. In fact, in most other higher plant chloroplast genomes studied (11-17) trnT and trnY are found on fragments that are fairly separated on the restriction map and, as can be seen on Figure 2, most of these repeats are found in the region between trnT and trnY. A similar situation was found in the intergenic region of two other rearranged chloroplast tRNA genes in broad bean (39) where the repeats, however, are significantly longer.

Chloroplast DNA codes for tRNAs specific for all 20 amino acids

In the higher plants studied so far, the genes for at most 17 amino acids have been located on the chloroplast genome. In broad bean, the unmapped tRNA genes are those specific for cysteine, glutamine and glutamic acid (15). The genes for $tRNA^{Glu}$ (this paper) and $tRNA^{Gln}$ (40) have been sequenced. The only gene on which no information is available in broad bean

chloroplasts is that for $tRNA^{Cys}$. But this gene has been sequenced in spinach chloroplasts (41) and it is very likely that it is also present in the chloroplast genomes from other plants. This means that the chloroplast genome codes for tRNAs capable of accepting all 20 amino acids. It remains to be seen, however, whether these chloroplast tRNAs (identified by mapping and sequencing the corresponding genes) contain all the anticodons required for protein synthesis according to the wobble hypothesis (42). In fact the genetic code used in chloroplasts is far from being completely known, although substantial information has been obtained from comparison of protein gene sequences with the sequences obtained by direct protein sequencing. One unusual feature was found so far in spinach chloroplasts, where the gene for an isoleucine tRNA has a methionine anticodon (43).

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