Variations in the Levels of Chloroplast tRNAs and Aminoacyl-tRNA Synthetases in Senescing Leaves of Phaseolus vulgaris

Chelliah Jayabaskaran, Marcel Kuntz, Pierre Guillemaut, and Jacques-Henry Weil

Institut de Biologie Moléculaire des Plantes du C.N.R.S., Université Louis Pasteur, 12 rue du Général Zimmer, 67084 Strasbourg, France

ABSTRACT

The relative amounts of chloroplast tRNAs^1, tRNAs^2, tRNAs^3, tRNAs^4, and tRNA^5 were determined in chloroplasts and cytoplasmic aminoacyl-tRNA synthetases in green leaves, yellowing senescing leaves, and N^6-benzyladenine-treated senescing leaves from bean Phaseolus vulgaris var Contender. Aminoacylation of the tRNAs using Escherichia coli aminoacyl-tRNA synthetases indicated that in senescing leaves the relative amount of chloroplast tRNA^6 was significantly lower than in green leaves. Senescing leaves treated with N^6-benzyladenine contained higher levels of this tRNA than untreated senescing leaves. No significant change in the relative amounts of chloroplast tRNAs^1-5, tRNAs^6, and tRNA^5 was detected in green, yellow senescing, or N^6-benzyladenine-treated senescing leaves. Relative levels of chloroplast tRNAs were also estimated by hybridization of tRNAs to DNA blots of gene-specific probes. These experiments confirmed the results obtained by aminoacylation and revealed in addition that the relative level of chloroplast tRNA^6 is higher in senescing leaves than in green leaves. Transcription run-on assays indicated that these changes in tRNA levels are likely to be due to a differential rate of degradation rather than to a differential rate of transcription of the tRNA genes. Chloroplastic and cytoplasmic leucyl-, phenylalanyl-, and tyrosyl-tRNA synthetase activities were greatly reduced in senescing leaves as compared to green leaves, whereas N^6-benzyladenine-treated senescing leaves contained higher enzyme activities than untreated senescing leaves. These results suggest that during senescence, as well as during senescence-retardation by cytokinins, changes in enzyme activities, such as aminoacyl-tRNA synthetases, rather than reduced levels of tRNAs, affect the translational capacity of chloroplasts.

Senescence is the final phase of growth and development. It is characterized by a decline in photosynthesis rate and electron transport activity (5). Chl catabolism (1), and changes in the levels of protein and RNA (5, 8, 14, 18, 23). Various models have been proposed to account for cellular aging. One of the models suggests that senescence and cell death is the result of a loss of translational capacities (22). Specifically, the model implies that changes occur in isoaccepting tRNA species and aminoacyl-tRNA synthetases. In plants, it has been shown that a loss of specific tRNAs occurs in chloroplasts during senescence of leaves (25). Recently, it was shown that in chloroplasts there is a correlation between the amounts of tRNAs specific for a given amino acid and the frequency of the codons specifying this amino acid (16). Furthermore, in the amino acids coded for by more than one codon, the population of isoaccepting tRNAs is adjusted to the frequency of the corresponding synonymous codons. Such an adjustment is probably necessary to ensure maximum efficiency in chloroplast protein biosynthesis (16). Thus, during senescence, limiting quantities of specific chloroplast tRNAs or aminoacyl-tRNA synthetases could restrict the chloroplast's capacity to read certain codons, and the resulting decrease in synthesis of essential cellular protein components would lead to deleterious effects on cell function.

It is well known that administration of cytokinins to senescing plants markedly delays leaf senescence and maintains or even increases the levels of RNAs and proteins (11, 12, 15, 24). In particular, it has been suggested that cytokinin treatment of plants at different stages of senescence could cause an increase in specific chloroplast tRNAs (4, 25).

To determine whether specific chloroplast tRNAs and whether chloroplastic or cytoplasmic aminoacyl-tRNA synthetases could act as limiting factors in senescing leaves, we measured the levels of chloroplast tRNAs and aminoacyl-tRNA synthetases in green, yellow senescing, and cytokinin-treated senescing leaves of bean. Furthermore, experiments were designed to study the transcriptional activity of chloroplast genes during senescence.

MATERIALS AND METHODS

Plant Material and Isolation of Plastids

Green, yellow senescing, and N^6-benzyladenine-treated senescing leaves from 2 month old bean Phaseolus vulgaris var Contender plants were used for these studies. The green leaves were of mature size that did not show any senescence symptoms. Senescing leaves (yellowing leaves still exhibiting, a light-green color around the midrib) were sprayed with 10^-4 M N^6-benzyladenine solution every 2 to 3 d and were harvested after three sprays. The portions of the treated leaves that turned from yellow to a visible pale-green color were used in this study. The control untreated leaves that were fully yellow and almost at the abscission stage were termed yellow senescing.
leaves in this report (at this stage, cytokinin treatment is no longer able to promote a re-greening process). Chloroplasts were purified from bean leaves as described previously (21).

**Preparations of tRNAs and Aminoacyl-tRNA Synthetases**

Total chloroplast tRNAs and total leaf aminoacyl-tRNA synthetases from green, yellow senescing, and N⁶-benzyladenine-treated senescing leaves were prepared according to Steinmetz and Weil (21). Aminoacylation of tRNAs was performed as described previously (21).

**Labeling of tRNAs**

For hybridization studies, total chloroplast tRNAs were treated with snake venom phosphodiesterase (Worthington, Freehold, NJ) to remove the terminal nucleotide(s) of the CCA end, were extracted with phenol/chloroform, and were enzymatically labeled at the 3'-end in the presence of α-[³²P] amino acid, α-[³²P] JCTP, and yeast tRNA nucleotidyl transferase (20). Transcription assays were performed as described (9) using isolated chloroplasts in the presence of α-[³²P] JUTP.

**Plasmids and Hybridizations**

Recombinant plasmids used were pVfC26, which contains BamHI fragment No. 19 (2.0 kb) from broad bean (*Vicia faba*) chloroplast DNA encoding the genes for tRNA<sup>Thr</sup> GU (trn T), tRNA<sup>Thr</sup> UUC (trn E), and tRNA<sup>Tyr</sup> GUA (trn Y) (10), and pVfCB53, which contains BamHI fragment No. 6a (5.3 kb) from broad bean chloroplast DNA encoding the genes for tRNA<sup>Leu</sup> CAA (trn L1), tRNA<sup>Leu</sup> UAG (trn L2), tRNA<sup>Leu</sup> UAA (trn L3), and tRNA<sup>Phe</sup> GAA (trn F) (7) were used. Hybridization of labeled tRNAs to DNA blots containing the above-mentioned plasmids cut with the appropriate restriction endonucleases was performed as described (13), and relative tRNA levels were determined by densitometric scanning of the autoradiograms using a Shimadzu CS-930 scanner and DR-2 peak-area measurer.

**RESULTS AND DISCUSSION**

**Relative Levels of tRNAs**

Transfer RNA preparations from bean chloroplasts of green, yellow senescing, and cytokinin-treated (N⁶-benzyladenine) senescing leaves (see “Materials and Methods”) were aminoacylated with leucine, phenylalanine, threonine, and tyrosine, using *Escherichia coli* or chloroplast enzymes. The relative amounts of tRNA(s) specific for one amino acid, measured by aminoacylation, was compared in the three tRNA samples (Table I). Significant differences were observed in the relative levels of tRNA<sup>Phe</sup>. When the amount of this tRNA was set arbitrarily to 100% in green leaves, the relative levels of tRNA<sup>Phe</sup> in yellow senescing leaves and N⁶-benzyladenine-treated senescing leaves were only 38 and 71%, respectively. In contrast, the relative amounts of tRNA<sup>Leu</sup>, tRNA<sup>Tyr</sup>, and tRNA<sup>Thr</sup> remained about the same in all three cases. The relative levels of individual chloroplast tRNAs, namely tRNA<sup>Asc</sup> UUC, tRNA<sup>Leu</sup> CAA, tRNA<sup>Leu</sup> UAG, tRNA<sup>Leu</sup> UAA, tRNA<sup>Phe</sup> GAA, tRNA<sup>Thr</sup> GU, and tRNA<sup>Tyr</sup> GUA, were estimated in green, yellow senescing, and N⁶-benzyladenine-treated senescing leaves by hybridization of [³²P]-labeled total chloroplast tRNAs to DNA blots containing gene-specific probes. These experiments (Fig. 1) confirmed that yellow senescing and N⁶-benzyladenine-treated senescing leaves contain reduced relative amounts of tRNA<sup>Phe</sup> with respect to green leaves and that this reduction appears less pronounced after N⁶-benzyladenine-treatment. The relative levels of the other tRNA tested appeared not to change significantly, as already suggested by the aminoacylation data, with the exception of tRNA<sup>Leu</sup> CAA (tRNA<sup>Leu</sup>) which is present at a slightly higher relative amount in green leaves. In addition, the results of these experiments (Fig. 1) showed that the relative level of tRNA<sup>Asc</sup> (which could not be measured by aminoacylation) is significantly higher in yellow senescing and N⁶-benzyladenine-treated senescing leaves than in green leaves. It has been established that in barley this tRNA<sup>Asc</sup> plays a dual role: the charged glutamate is used in protein synthesis and also provides the activated substrate for the dehydrogenase involved in Δ-aminoenolulinate biosynthesis (19), which is a precursor of Chl.

In bean chloroplasts, two tRNA<sup>Phe</sup> isoacceptors were identified (6). It is interesting to note that both isoacceptors contain cytokinin-like modified nucleosides: the major tRNA<sup>Phe</sup> isoacceptor contains 2-methyl-thio-N⁶-isopentenyladenosine (m2-PA) (6) and the minor tRNA<sup>Phe</sup> isoacceptor contains either N⁶-isopentenyladenosine (PA) or the modified form (m2-PA) adjacent to the anticodon (our unpublished results). Anderson and Cherry (2) and Bick et al. (3) have shown that administration of another cytokinin, namely N⁶-benzyladenine, to soybean cotyledons and hypocotyls alters the relative levels of two or more tRNA<sup>Leu</sup> isoacceptor species. To explain the senescence-retarding effects of cytokinins, such as N⁶-benzyladenine on zeatin, or chloroplast tRNAs, a model has been proposed (4) that is based on the hypothesis that a specific nucleoside is able to cleave PA-containing tRNAs. The degradation of the PA-containing tRNAs would then reduce the synthesis of certain proteins during plant senescence. In contrast, cytokinin-treated tissues would retain their PA-containing tRNAs and would continue to synthesize proteins. Our data, and more particularly the variation in the relative amounts of tRNA<sup>Phe</sup> during senescence, could be explained partly by this model, but since preliminary results (not shown) indicate that the variation in the relative levels of some tRNAs is due, at least to some extent, to degradation at the 3'-end, it is likely that other mechanisms are also involved.

**Transcription of tRNA Genes**

Since variations of the relative levels of tRNAs could also be due to differential transcription of the tRNA genes, experiments were designed to study the transcriptional activity of chloroplast tRNA genes during senescence. Transcription run-on assays were performed with chloroplasts isolated from green, yellow senescing, and N⁶-benzyladenine-treated senescing leaves and α-[³²P] JUTP-labeled run-on tRNAs were hybridized to Southern blots of gene-specific probes. Transcription of all tested tRNA genes was detected in green leaves.
Table 1. Relative Levels of Bean Chloroplast tRNAsLeu, tRNA^{Asp}, tRNA^{Thr}, and tRNA^{Tyr} from Green, Yellow Senescing, and N^6-Benzyladenine-Treated Senescing Leaves Measured by Aminoacylation

<table>
<thead>
<tr>
<th>Type of Leaves</th>
<th>Relative Amounts of tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tRNA^{Leu} (L)</td>
</tr>
<tr>
<td>Green</td>
<td>100</td>
</tr>
<tr>
<td>(1.7–2.2)</td>
<td>(0.76–1.05)</td>
</tr>
<tr>
<td>Yellow senescing</td>
<td>91</td>
</tr>
<tr>
<td>N^6-Benzyladenine-treated senescing</td>
<td>97</td>
</tr>
</tbody>
</table>

Figure 1. A. Hybridization of total chloroplast ^32P-labeled tRNAs with DNA probes specific for genes encoding tRNA^{Asp}CCA (trnL1), tRNA^{Asp}UAG (trnL2), tRNA^{Asp}UAA (trnL3), tRNA^{Asp} (trnE), tRNA^{Thr} (trnF), tRNA^{Thr}GGU (trnT), and tRNA^{Tyr} (trnY). Plasmids containing the gene-specific probes (see "Materials and Methods") were digested with restriction endonucleases (lane 1: pVicB53 was digested with BamHI, HaellII, and XbaI; lane 2: pVic26 was digested with EcoRI and Sallin in order to produce DNA fragments containing a single tRNA gene. These DNA fragments were separated by electrophoresis on a 1.5% agarose gel, transferred onto Hybond-N (Amersham) membranes and hybridized with chloroplast tRNAs isolated from green (G), yellow senescing (Y), and cytokinin-treated senescing (C) leaves. B. Relative amounts of the above-mentioned tRNAs determined by densitometric scanning of the autoradiograms. The sum of the densitometric values obtained for the seven tRNAs in a given tissue was set as 100.

Figure 2. Hybridization of chloroplast run-on transcripts with DNA probes specific for genes encoding tRNA^{Asp}CCA (trnL1), tRNA^{Asp}UAG (trnL2), tRNA^{Asp}UAA (trnL3), tRNA^{Asp} (trnE), tRNA^{Thr} (trnF), tRNA^{Thr}GGU (trnT), and tRNA^{Tyr} (trnY). Plasmids containing the gene-specific probes (lane 1 and 2) were processed as described in Figure 1. Chloroplast transcription assays were performed in presence of ^32PjUTP as described (9). Experiments performed using purified chloroplasts from green (G) and cytokinin-treated senescing (C) leaves are shown.

(Fig. 2) but not in yellow leaves and N^6-benzyladenine-treated senescing leaves (Fig. 2). It is difficult to draw firm conclusions from this negative result. However, these data suggest that the tRNAs identified in yellow senescing leaves and in N^6-benzyladenine-treated senescing leaves are mainly pre-existing tRNAs and not de novo synthesized tRNAs, and that the variation in the relative levels of these tRNAs during senescence is mainly due to differential rates of degradation. However, it is possible that the effect on tRNA levels is due to early suppression of transcription for some tRNA genes (e.g., tRNA^{Asp}) and late suppression for other genes during the onset and progression of senescence. It is also noteworthy that cytokinin treatment apparently did not lead to a detectable increase in transcription of chloroplast tRNA genes in senescing leaves.

Activities of Aminoacyl-tRNA Synthetases

To determine whether or not chloroplastic and cytoplasmic leucyl-, phenylalanyl-, threonyl-, and tyrosyl-tRNA synthetase activities differ in green, yellow senescing, and N^6-benzyladenine-treated senescing leaves, tRNA samples from cytoplasm
(isolated from hypocotyls) and chloroplasts were charged using an enzyme preparation from the various types of leaves. Figure 3 clearly shows the different activities of these three aminoacyl-tRNA synthetase preparations. In yellow senescing leaves, the activities of both cytoplasmic and chloroplastic leucyl- and tyrosyl-tRNA synthetases were extremely low, whereas in N⁶-benzyladenine-treated senescing leaves these enzyme activities were significantly higher and were even higher in green leaves. The activities for both cytoplasmic and chloroplastic phenylalanyl-tRNA synthetases were high in green leaves and low, but clearly detectable, in yellow senescing leaves and in N⁶-benzyladenine-treated senescing leaves; in this case, cytokinin-treatment did not cause higher enzyme activities. This is also the case for cytoplasmic and chloroplastic threonyl-tRNA synthetase activities, which remained relatively high in yellow senescing leaves.

The decreasing activities of some chloroplastic and cytoplasmic tRNA synthetases in bean leaves during senescence could lead to a lower efficiency of the synthesis of proteins required for chloroplast functioning and may result in an inhibition of chloroplast biogenesis. These results indicate that aminoacyl-tRNA synthetases could act as a limiting factor in senescence. The low levels of some aminoacyl-tRNA synthetases (including the cytosolic enzymes) at the stage we refer to as yellow senescing leaves (see "Materials and Methods") suggest that protein synthesis does not occur any longer at this stage.

It is well established that administration of cytokinin to senescing cotyledons or leaves from different plant species results in a rapid increase in protein synthesis (24) and a decrease in protein breakdown (11). Increased synthesis of specific enzymes caused by cytokinins has also been reported (12). The results obtained in our studies show that N⁶-benzyladenine rapidly stimulates leucyl- and tyrosyl-tRNA synthetase activities in cytokinin-treated senescing leaves.

CONCLUSIONS

In higher plants, changes in tRNAs and synthetases have been shown to correlate with growth, development, and senescence (2, 3, 17, 25). The results obtained in our studies suggest that the limiting quantities of specific tRNAs, such as chloroplast tRNA⁸⁶⁷, can affect the protein synthesis capacity of chloroplasts during senescence. However, our data demonstrating a dramatic reduction in enzymatic activity for some aminoacyl-tRNA synthetases during senescence suggest that this reduction in enzymatic activity restricts the capacity to read certain codons in the chloroplast during senescence more severely than does the reduction in the amounts of some tRNAs. In agreement with this model is the fact that cytokinin treatment, which leads to a transitory and partial regreening process in the senescing leaves, induces an increase in those aminoacyl-tRNA synthetase activities which are very low in yellow senescing leaves, without a detectable increase in the transcription rate of the tRNA genes.

ACKNOWLEDGMENTS

We wish to thank A. Cosset and M. Arborgast for the skilled technical assistance. We also thank F. Herdenberger for plasmid DNA pVFcB53.

LITERATURE CITED