Identification and Structural Characterization of Nucleus-encoded Transfer RNAs Imported into Wheat Mitochondria*

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Despite its large size (200–2400 kilobase pairs), the mitochondrial genome of angiosperms does not encode the minimal set of tRNAs required to support mitochondrial protein synthesis. Here we report the identification of cytosolic-like tRNAs in wheat mitochondria using a method involving quantitative hybridization to distinguish among three tRNA classes: (i) those encoded by mitochondrial DNA (mtDNA) and localized in mitochondria, (ii) those encoded by nuclear DNA and located in the cytosol, and (iii) those encoded by nuclear DNA and found in both the cytosol and mitochondria. The latter class comprises tRNA species that are considered to be imported into mitochondria to compensate for the deficiency of mtDNA-encoded tRNAs. In a comprehensive survey of the wheat mitochondrial tRNA population, we identified 14 such imported tRNAs, the structural characterization of which is presented here. These imported tRNAs complement 16 mtDNA-encoded tRNAs, for a total of at least 30 distinct tRNA species in wheat mitochondria. Considering differences in the set of mtDNA-encoded and imported tRNAs in the mitochondria of various land plants, the import system must be able to adapt relatively rapidly over evolutionary time with regard to the particular cytosolic-like tRNAs that are brought into mitochondria.

In flowering plants (angiosperms), all of the codons of the canonical genetic code are represented in the protein genes encoded by mtDNA, yet the mitochondrial genome appears to specify fewer than the 23–24 or 33 tRNAs minimally required to read these codons by either expanded or standard wobble base pairing, respectively. This observation, coupled with the identification of cytosolic-like tRNAs in mitochondrial RNA preparations of bean (1), wheat (2), and potato (3), suggests that tRNAs must be imported into angiosperm mitochondria (4–6), as they are into the mitochondria of many unicellular eukaryotes (7). A requirement for mitochondrial tRNA import in plants is rather surprising in view of the large size of the angiosperm mitochondrial genome (200–2400 kilobase pairs), which in principle could easily accommodate a full set of tRNA genes, as do the much more compact mtDNAs of most animals and fungi (8).

Depending on their genome of origin, plant mitochondrial tRNAs (mt-tRNAs) can be divided into three groups (see Refs. 2 and 4–6): (i) native mt-tRNAs, encoded by plant mtDNA and presumed to originate from the eubacteria-like endosymbiont that was the evolutionary source of the mitochondrion and its genome; (ii) chloroplast-like tRNAs, also encoded by plant mtDNA but originating from promiscuous chloroplast DNA sequences that in the course of evolution have been transferred to and are now an integral part of the mitochondrial genome; and (iii) imported tRNAs, encoded by nDNA and localized in mitochondria as well as the cytosol. Given that tRNAs encoded by a particular genome within a plant cell may function in more than one subcellular compartment, complete characterization of a plant tRNA requires not only determination of its anticodon sequence and aminoacylation specificity, but also its intracellular localization and that of the gene encoding it. Although the set of mtDNA-encoded tRNAs or their genes has been examined in a variety of angiosperms (3, 9, 10), only in potato (a dicotyledonous plant) has the mt-tRNA population been extensively characterized (3). In that plant, 31 mt-tRNAs were found, including 20 encoded by mtDNA (15 native and 5 chloroplast-like) and 11 specified by nDNA.

The research reported here aimed to identify and comprehensively characterize nDNA-encoded tRNAs that are imported into wheat mitochondria from the cytosol. The possibility of mitochondrial tRNA import emerged when studies to catalog wheat mtDNA-encoded tRNA genes revealed only 16 genes specific for 13 amino acids (2). Although we considered that some mtDNA-encoded tRNA genes might have escaped detection in this survey, it seemed unlikely that all of the remaining ~17 tRNA genes required by standard wobble rules could have been missed. The apparently limited number of wheat mtDNA-encoded tRNAs prompted a preliminary investigation of the wheat mitochondrial tRNA population, which identified three cytosolic-like tRNA species (2); however, the latter study did not address the issue of possible cytosolic tRNA contamination of the wheat mitochondrial tRNA preparation. In the present investigation, we systematically examined the question of preparative contamination and established criteria and procedures for the identification of tRNAs that are genuinely imported into wheat mitochondria.

**EXPERIMENTAL PROCEDURES**

**Isolation of Mitochondria**—The procedures of Spencer et al. (11) were used for preparation of viable wheat embryos (from pedigree seed of *Triticum aestivum* var. Katepwa; Alberta Wheat Pool, Calgary, Alberta, Canada), germination of embryos and isolation of mitochondria.

**Isolation of Mitochondrial and Nuclear DNA**—Wheat mtDNA was prepared from DNase-treated, sucrose gradient-purified mitochondria (11) by sequential sarkosyl lysis, incubation of the lysate with Pronase,
and recovery of DNA by centrifugation in CsCl-ethidium bromide gradients (11, 12). Wheat nDNA was isolated in a similar fashion from a crude nuclear fraction that was further purified in a discontinuous sucrose gradient (11), except that nuclei were extracted with a phenol/creosol mixture rather than being treated with Pronase.

Fractionation of Wheat Mitochondrial tRNAs by Polyacrylamide Gel Electrophoresis—To obtain a sufficient quantity of individual tRNAs for subsequent analysis, a two-dimensional gel electrophoresis procedure (13) was adapted. Gel solutions and electrophoretic conditions were as described previously (11). For isolation of total cellular RNA, germinating embryos were ground in a mortar with equal volumes of 0.05 M Tris-HCl, 0.02 M Na-EDTA (pH 8.0) and phenol/creosol mix. The homogenate was centrifuged at 18,000 × g for 20 min, an equal volume of phenol/creosol solution was added, and the mixture was shaken for 10 min at 4 °C and then centrifuged at 18,000 × g for 10 min. After addition of NaCl to 0.5 M to the aqueous phase, RNA was purified by repeated phenol extraction and ethanol precipitation.

Fractionation of Wheat Mitochondrial tRNAs by Polyacrylamide Gel Electrophoresis—Two-dimensional gel electrophoresis (20) was adapted. Gel solutions and electrophoretic conditions were as described above), and washing of blots were conducted as described (14). blots were exposed to Kodak X-Omat K x-ray film without intensifying screens, the resulting autoradiographs were digitized, the intensity of each band was determined using the NIH Image Analysis program, and linear regression coefficients were calculated using MacCurveFit.

Polymerease Chain Reaction (PCR) Amplification and Cloning of Wheat Nuclear tRNA Genes—PCR amplification experiments employed oligonucleotides (generally 20-mers) designed to target the 5'- and 3'-terminal regions of the desired tRNA gene, using sequence information obtained by chemical and RT sequencing of the corresponding tRNA. Nuclear-encoded tRNA sequences (DNA and RNA) of other plants (21) assisted in primer design.

Purified nDNA (11) was used either directly or after extensive shearing by two passes at 20,000 p.s.i. through a French pressure cell. The sheared DNA (mean size about 400 base pairs) produced few, if any, artifactual bands and so was the preferred template. Amplification was in a Perkin Elmer GeneAmp PCR System 2400 using Vent (exo -) DNA polymerase (New England Biolabs) with 500 ng of nDNA. An optimized regime employed 40 cycles and included a 30-s annealing at 55 °C and a 30-s extension at 72 °C, with the ramp-up from annealing to extension slowed to 50% of maximum. PCR was also attempted with 5% (v/v) dimethyl sulfoxide or 1.3 M betaine in the reaction mix; however, neither reagent supported the amplification of tRNA sequences that were not also recovered using the standard protocol.

For cloning, blunt-ended PCR products were ligated into pT7 Blue® (Novagen) and transformed into DH5α cells that had been prepared by the protocol of Inoue et al. (22). Sequence was determined using the fmol Cycle Sequencing® kit (Promega) with 5'-32P-labeled vector-based primers.

RESULTS

The term "mitochondrial tRNA" (mt-tRNA) is used here to refer generally to tRNA species present in the isolated mitochondrial tRNA population, whether these species are encoded by the mitochondrial genome (mtDNA-encoded mt-tRNA) or nuclear genome (nDNA-encoded mt-tRNA). The term "cytosolic tRNA" (cy-tRNA) is reserved for nDNA-encoded tRNAs that are normally localized to and function in the cytosol. The term "imported tRNA" is applied to those nDNA-encoded tRNAs that are selectively accumulated in mitochondria, presumably for use in mitochondrial protein synthesis.

Preliminary Experiments—When radioactively end-labeled wheat mt-tRNAs were incubated with mtDNA or nDNA, only a fraction of the total mt-tRNA population hybridized to mtDNA. After two-dimensional polyacrylamide gel electrophoresis, the latter tRNAs generated a distinctly different pattern than those hybridizing to nDNA (23). Among the latter group were three cytosolic-type tRNAs (Gly (CCC); Leu (CAA); Val (GAC); anticodon sequences in parentheses) previously identified in the wheat mt-tRNA population (2). Total cy-tRNA had a more complex gel electrophoretic profile than the mt-tRNA fraction hybridizing to nDNA, and it contained unique species (23). This makes it unlikely that cy-tRNA contamination of the mt-tRNA fraction is of such magnitude and extent that those mt-tRNAs that hybridize to nDNA represent contaminating cy-tRNAs, rather than bona fide imported tRNAs. Nevertheless, this approach cannot unequivocally distinguish imported nDNA-encoded tRNAs from nDNA-encoded cy-tRNAs contaminants of the mitochondrial preparation.

To assess the level of cy-tRNA co-isolating with wheat mitochondrial RNA, a wheat cytosolic RNA preparation was incubated with nDNA and nDNA, only a fraction of the total mt-tRNA population hybridized to mtDNA. After two-dimensional polyacrylamide gel electrophoresis, the latter tRNAs generated a distinctly different pattern than those hybridizing to nDNA (23). Among the latter group were three cytosolic-type tRNAs (Gly (CCC); Leu (CAA); Val (GAC); anticodon sequences in parentheses) previously identified in the wheat mt-tRNA population (2). Total cy-tRNA had a more complex gel electrophoretic profile than the mt-tRNA fraction hybridizing to nDNA, and it contained unique species (23). This makes it unlikely that cy-tRNA contamination of the mt-tRNA fraction is of such magnitude and extent that those mt-tRNAs that hybridize to nDNA represent contaminating cy-tRNAs, rather than bona fide imported tRNAs. Nevertheless, this approach cannot unequivocally distinguish imported nDNA-encoded tRNAs from nDNA-encoded cy-tRNAs contaminants of the mitochondrial preparation.
to polyacrylamide gel electrophoresis. No differences in the electrophoretic banding or intensity patterns of the radiolabeled cy-tRNA associated with the mitochondrial fraction could be discerned in comparison to those of similarly labeled cy-tRNA fractions (23).

Micrococcal nuclease treatment of isolated mitochondria has been used in other studies to reduce contamination of mitochondrial RNA preparations by cytosolic tRNA (24, 25). To assess the effectiveness of this treatment, we carried out a quantitative investigation (23), which showed that such treatment reduced the amount of radiolabeled cy-tRNA co-isolating with mitochondrial RNA by at least 50% compared with untreated mitochondria. However, as in other published work, the yield of mitochondrial RNA was also substantially reduced. This meant that, on average, micrococcal nuclease treatment lowered residual contamination by approximately 35%, not 50%.

To ascertain whether the cy-tRNA remaining after micrococcal nuclease treatment was a specific subset of the cy-tRNA population, the mitochondrial RNA was electrophoresed in a 4 M urea, 10% polyacrylamide gel. Although the signal was faint, no selectivity was evident (23).

**Determination of the Subcellular Location of Wheat tRNAs—** Because micrococcal nuclease treatment of wheat mitochondria was not effective in reducing the amount of added cy-tRNA that co-isolated with mt-tRNA to what we would consider a negligible level (<5% of total mt-tRNA), we therefore developed an alternative approach for distinguishing bona fide, imported tRNAs from ones nonspecifically associated with mitochondria, possibly as a result of preparative contamination by cy-tRNAs. Slot blots containing mitochondrial RNA and cytosolic RNA were hybridized with oligonucleotides (Table I) specific for mtDNA-encoded tRNAUGG (26), the cytosolic-like tRNA\(^{\text{Gly}}\)GCC (27) previously identified in wheat mitochondrial RNA (2), and nDNA-encoded cy-tRNA\(^{\text{Pro}}\)GCC (28). The latter tRNA is not required in wheat mitochondria (i.e. is functionally redundant) because the mtDNA encodes a tRNA\(^{\text{Pro}}\) (2).

As expected, distinctly different hybridization patterns were obtained in these experiments (Fig. 1). With the tRNA\(^{\text{Pro}}\)GCC oligonucleotide, there was a strong signal with mitochondrial RNA and a much weaker signal with cytosolic RNA. The reverse was seen with the tRNA\(^{\text{Pro}}\)GCC oligonucleotide: a strong signal with cytosolic RNA and a much weaker signal with mitochondrial RNA. With tRNA\(^{\text{Gly}}\)GCC (a nDNA-encoded tRNA previously considered to be imported into wheat mitochondria (Ref. 2), the corresponding oligonucleotide hybridized equally well with mitochondrial and cytosolic RNA.

To quantify the relative intensities of the hybridization signals, autoradiographs were digitized and the relationship between the strength of each signal and RNA concentration was determined in the linear region of the plot. The slopes of the lines (regression coefficients) obtained following hybridization of an oligonucleotide to mitochondrial RNA and cytosolic RNA were then compared, providing an estimate of the relative intensities of the two signals (Table II).

The ratios (mitochondrial RNA/cytosolic RNA) of the regression coefficients (RRC) for tRNA\(^{\text{Gly}}\)GCC and another cy-tRNA, tRNA\(^{\text{Pro}}\)GCC (also not required by the mitochondria because wheat mtDNA encodes a tRNA\(^{\text{Pro}}\) (Refs. 29 and 26), were only 0.24 and 0.31, respectively, compared with an RRC of 8.3 for tRNA\(^{\text{Pro}}\)GCC. As expected, the RRC for tRNA\(^{\text{Gly}}\)GCC was different than in the case of the other two classes of tRNA, being close to 1 (1.35). This method provides a relative measure of the concentration of each tRNA in the cytosolic and mitochondrial fractions and offers a way to distinguish nDNA-encoded tRNAs that specifically accumulate in mitochondria (i.e. imported tRNAs, by our definition) from those cy-tRNAs that may be present solely as a result of contamination.

This slot blot procedure was used to assess the import status of 16 cytosolic-like wheat mt-tRNAs isolated by two-dimensional gel electrophoresis, as described in the next section. Sequence analysis of individual tRNAs provided information for synthesis of tRNA-specific oligonucleotide probes (Table I). Discussion of the import status of these tRNA species follows below, after presentation of data relating to their structural characterization.

**Isolation and Sequencing of Wheat Mitochondrial tRNAs—** To isolate individual tRNAs, we adopted a procedure involving two-dimensional polyacrylamide gel electrophoresis of a relatively large quantity (125 \(\mu\)g) of a 1 M NaCl-soluble fraction of wheat mitochondrial RNA (~85% tRNA). Fig. 2 shows the resulting ethidium bromide-stained RNA profile. Heterogeneity at the metabolically labile 3’-CCA\(_{\text{OH}}\) terminus (all or a portion of which may be lacking in an individual tRNA) was anticipated (30) and, as confirmed by sequence analysis, is particularly evident at the periphery of the RNA profile. In general, a single isoaccepting tRNA was resolved into three species by this procedure.

**Spots numbered 1–50 in Fig. 2** correspond to tRNAs in the two-dimensional gel electrophoretic pattern previously reported in Ref. 2. Prior to electrophoresis in that study, wheat mt-tRNAs were 3’-end-labeled using wheat tRNA nucleotidyl-transferase, thereby eliminating the 3’-end heterogeneity observed here. **Lowercase letters in Fig. 2** denote putative single tRNA species with varying degrees of completion of the 3’-terminal -CCA\(_{\text{OH}}\)Bands numbered 70–79 do not appear to match any of those reported in Ref. 2.

Following the initial two-dimensional polyacrylamide gel fractionation, recovered tRNAs were radiolabeled and electrophoresed in a denaturing polyacrylamide gel (“third dimension”) (Fig. 3). Many of the tRNAs isolated from the initial two-dimensional gel were homogeneous at this stage, whereas others separated into two, three, and occasionally four bands. This result is primarily a consequence of overlap of species in the two-dimensional gel because of 3’-end heterogeneity, although it is known that individual tRNAs can exist in distinct, separable forms due to variation in post-transcriptional modifications (31). Species resolved in the third dimension are iden-

**Table I**

| tRNA\(^{\text{a}}\) | Complementary position in tRNA sequence \((\beta \rightarrow \gamma)\) | Oligonucleotide sequence |
|------------------|---------------------------------|--------------------------|
| Phe (GGA)        | 72–53                           | CAAGCTAGGTCGGAGATTGCGG   |
| Asp (GUC)        | 73–54                           | CGGCGTGCAGGGGGATCAGAA    |
| Pro (UGG)        | 73–54                           | TCAGAATGGACACGTTCTGCGA   |
| Ala1 (IGC)       | 71–55                           | TGGCGATGTTGCGGGATTGCGA   |
| Ala2 (UGC)       | 69–55                           | GTAAGGGGATGTTGCGGAA      |
| Ala3 (IGC)       | 71–55                           | GAGTGGCGGGGAAATTGCGA     |
| Arg1 (IGC)       | 73–54                           | CGACTCCGCTGGGGATCAGAA    |
| Arg2 (CCU)       | 72–53                           | GGCACAGTGGGATGTTGCGA     |
| Gly1 (GCC)       | 50–30                           | GGGCTGGCAGACCGTTGCGA     |
| Gly2 (UCC)       | 73–54                           | TGGCTGTTGCGGGATTGCGA     |
| His (GUG)        | 70–51                           | GGGCTGTTGCGGGATTGCGA     |
| Ile (IAC)        | 71–52                           | GCCCTACAGGTTCTGCGA       |
| Leu1 (UAG)       | 72–53                           | ACAGAATGGGATGTTGCGA      |
| Leu2 (IAG)       | 72–53                           | GACACCTGTTGCGGGATTGAC    |
| Leu3 (C*AA)      | 72–53                           | TCAGAATGGGATGTTGCGA      |
| Leu4 (U*AA)      | 72–53                           | CTGCTGTTGCGGGATTGCGA     |
| Val (CAG)        | 73–54                           | TGGCTGTTGCGGGATTGCGA     |
| Val (IAC)        | 71–52                           | TGCAGGCGGGGAGTTGCGA      |

\(^{\text{a}}\) Assigned anticodon sequences (see Table III) are shown in parentheses. Except for Phe (GAA) (28) and Pro (UGG) (26), tRNA sequences are displayed in Fig. 4.

\(^{\text{b}}\) Encoded by mtDNA (26).
we attribute to the presence in the variable loop of an unidentified modified nucleoside with greatly enhanced reactivity in the C reaction. Virtually complete scission of the polynucleotide chain occurred at the residue in question, with the result that very faint or no bands appeared in the C track of sequencing films beyond (5’ of) this position.

Except for tRNA\textsubscript{GCC}\textsuperscript{32P}*, all of the tRNAs listed in Table III were also sequenced using RT. In addition to confirming chemical sequence data, RT analysis was particularly useful in establishing sequence in the variable loop (a region often containing a number of unassigned nucleosides in the chemical sequence analysis) and in allowing identification of C residues unable to be assigned on the basis of the chemical sequence analysis because of the anomalous C-specific cleavage noted above. As well, RT sequencing confirmed or expanded anticodon sequence information for six of the tRNAs (Table III).

Thin-layer Chromatography—In chemical sequencing reactions the modified nucleoside inosine (I) is cleaved in the G-specific reaction, generating a band in the G lane. Inosine is present in the first position of the anticodon (position 34, the only site at which this modification occurs in tRNA; Ref. 21) in several plant tRNAs, including wheat germ tRNA\textsubscript{IAC} (32), potato mt-tRNA\textsubscript{IAC} (33), and lupin cy-tRNA\textsubscript{IAC} (34). In the present study, the sequence of the anticodon loop region of the Arg2 tRNA (anticodon “GCG; Table III) was directly determined (see “Experimental Procedures”), showing that inosine occupies the first (wobble) position of the anticodon of this tRNA (23), as documented independently elsewhere (33).

PCR Amplification of Nuclear tRNA Genes—PCR was used in an attempt to amplify nuclear gene sequences corresponding to all of the 16 cytosolic-like mt-tRNA species characterized here by RNA sequencing. This approach was successful in 11 cases (Table III), with sequence data from cloned PCR products either confirming the anticodon sequence or clarifying ones remaining ambiguous after RNA sequencing. Although an exhaustive attempt was made to recover the remaining five nuclear tRNA genes using a variety of amplification conditions and primer combinations, PCR products were not obtained in these cases.

Nucleotide Sequence Comparisons—The wheat mt-tRNAs described in this report were identified as cytosolic-like by comparison (35) with homologous mt- and cy-tRNA sequences from various organisms (23). In general, each wheat mt-tRNA identified here proved to be substantially more similar to a corresponding plant cy-tRNA or nuclear tRNA gene sequence (>80% identity) than to a plant mtDNA-encoded one (<60%),

### Table II

| tRNA* | Linear regression coefficientsa | RRCb |
|-------|---------------------------------|------|
|       | mRNA                            | cyRNA |      |
| Phe (GAA)c | 591                             | 2,747 | 0.24 |
| Asp (GUC)d | 3,121                           | 10,102| 0.31 |
| Pro (UGG)e | 5,868                           | 707   | 8.30 |
| Ala1 (IGC) | 2,214                           | 1,235 | 1.72 |
| Ala2 (UGC) | 7,043                           | 6,680 | 1.05 |
| Ala3 (IGG) | 6,807                           | 6,952 | 0.98 |
| Arg1 (ICG) | 3,290                           | 3,509 | 0.94 |
| Arg2 (CUC) | 8,640                           | 9,483 | 0.91 |
| Gly1 (GCC) | 9,786                           | 7,216 | 1.35 |
| Gly2 (UCC) | 6,246                           | 6,001 | 1.04 |
| His (UGU) | 2,754                           | 2,727 | 1.01 |
| Ile (IAU) | 25,266                          | 14,426| 1.75 |
| Leu1 (UAG) | 5,963                           | 3,985 | 1.50 |
| Leu2 (IAG) | 2,010                           | 4,297 | 0.47 |
| Leu3 (C*AA) | 3,031                          | 2,670 | 1.14 |
| Leu4 (U*AA) | 17,733                          | 3,633 | 4.88 |
| Val1 (CAC) | 14,125                          | 13,328| 1.06 |
| Val2 (IAC) | 13,292                          | 13,291| 1.00 |

a Sequences of wheat cytosolic-like tRNA species are shown in Fig. 4. Assigned anticodon sequences (see Table III) are enclosed in parentheses.
b Correlation coefficients (R\textsuperscript{2}) for the regression analyses ranged from 0.96 to 0.99.
c mtRNA/cyRNA.
d Nuclear DNA-encoded cy-tRNAs for which cognate tRNAs are encoded in mtDNA (26).
e Mitochondrial DNA-encoded tRNA species (26).

### Fig. 1.

Slot blot analyses of three wheat tRNA-specific oligonucleotide probes hybridized to wheat mitochondrial RNA and cytosolic RNA. Autoradiographs show the hybridization of 5'-\textsuperscript{32P}-labeled oligonucleotides specific for mtDNA-encoded mt-tRNA\textsubscript{GCC} (A), cy-tRNA\textsubscript{GCC} (B), and nDNA-encoded mt-tRNA\textsubscript{GCC} (C), to wheat mitochondrial RNA (M), wheat cytosolic RNA (C), total cellular RNA that had been treated with DNase I (I), and cytosolic RNA that had been treated with DNase I (C). RNA samples were serially diluted 2-fold to produce amounts ranging from 6 µg to 0.047 µg and applied to wells of the slot blot apparatus as described under “Experimental Procedures.”

The wheat mt-tRNAs identified here proved to be substantially more similar to a corresponding plant cy-tRNA or nuclear tRNA gene sequence (>80% identity) than to a plant mtDNA-encoded one (<60%),
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in cases where such mtDNA-encoded counterparts are available for comparison (23).

Primary and Secondary Structures of Sequenced tRNAs—The 16 wheat tRNA sequences determined here (Table III) can be folded into the standard cloverleaf secondary structure (Fig. 4). With few exceptions (23), these structures contain the expected invariant and semi-invariant nucleosides characteristic of a conventional tRNA.

For each wheat mt-tRNA sequence reported here, BLASTN searches (35) identified a number of highly similar plant homologs that allowed assignment of most of the remaining undetermined positions in the wheat sequences, and which provided sufficient additional information to permit inference of the anticodon sequence in all cases.

Alanine—Species Ala1 and Ala3 differ slightly in nucleotide sequence, but we conclude that they have the same anticodon, IGC. PCR amplification provided evidence of heterogeneity (C/T) at one position in the variable loop, and homologous Arabidopsis sequences with the corresponding heterogeneity were identified in BLAST searches (Table III and Fig. 4). PCR amplification also demonstrated that anticodon loop positions 34 and 37 are both A in the gene sequence, as they are in the corresponding Arabidopsis and other plant tRNAAla sequences. Positions 34 and 37 are inosine and 1-methylinosine (m1I) in sequenced cytoplasmic tRNAAla species, which would account for the fact that these residues registered as G during sequencing of wheat Ala1. Like their Arabidopsis counterparts, the wheat Ala1 and Ala3 sequences have A at position 54, rather than the almost universally conserved 5-methyluridine (m5U).

We infer that species Ala2 (ugN) has the anticodon sequence UGC, based on comparison with an Arabidopsis tRNAUGC sequence that differs by one compensated base pair in the T stem (Table III and Fig. 4). Because position 37 is A rather than G in the Arabidopsis gene sequence, it is likely that this position is occupied by m1I in the wheat Ala2 tRNA, as we suggest above for the Ala1 and Ala3 tRNAs.

Arginine—The Arg1 and Arg2 tRNA species are essentially identical to previously sequenced wheat tRNA Arg species with anticodons CCU (36) and ICG (33), respectively. PCR amplification of the wheat Arg2 sequence revealed the presence of wobble position A; as noted above, direct analysis identified this residue as inosine, as in the published sequence (33).

Aspartate—This sequence is identical to an Arabidopsis tRNAAsp sequence also having the anticodon GUC (Table III).

Glycine—The sequences of Gly1 and Gly2 are identical, respectively, to those of a previously published wheat tRNA GCC Gly (27) and an Arabidopsis homolog with anticodon UCC (Table III). Both Gly1 and Gly2 appear to have unmodified U rather than the usual m5U at position 54, as noted previously for wheat tRNAGC Gly (37).

Histidine—The wheat sequence differs at six positions from a sequenced lupin tRNAHis (38) but at only two positions from several Arabidopsis tRNAHis homologs (Table III and Fig. 4). Curiously, all of the Arabidopsis tRNAHis sequences currently in the data base (representing genes on several different chromosomes) have C rather than T at position 54, which is normally m5U in the mature tRNA. Both the wheat and lupin tRNAHis evidently contain unmodified U at this position.

Isoleucine—This tRNA sequence differs at several positions from a published wheat tRNAIle sequence (32). Within the D loop, the stretch AGUGG in our sequence is AGDDGG in the published wheat germ sequence and AG(C/T)TGG in homolo-
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### Table III

| Spot no. | Amino Acid | Chemical sequencing | RT sequencing | PCR | Assigned | Reference sequence |
|----------|------------|---------------------|---------------|-----|----------|-------------------|
| 18, 19, 20a.1 | Ala1 | GGc | " | AGC<sup>a</sup> | IGC | A.t. AL096882; 76788 (com) |
| 25.2 | Ala2 | ugN | " | " | UGC | A.t. AC006284; 67507 (com) |
| 42.2 | Ala3 | NGN | " | AGC<sup>a</sup> | IGC | A.t. AC004689; 14531 (com) |
| 24.1, 39a.1, 39b, 44a, 44b.1 | Arg2 | GCC | " | AGG<sup>b</sup> | IGC | T.a. tRNA<sub>ARG</sub> (Ref. 30) |
| 42.1 | Arg1 | NNU | CCU | CCU | CCU | T.a. tRNA<sub>ARG</sub> (Ref. 36) |
| 75.3 | Asp | GUC | " | " | GUC | A.t. AC013354; 30860 (com) |
| 10a, 10b, 23 | Gly1 | GCC | " | GGC | GCC | T.a. tRNA<sub>Glu</sub> (Ref. 27) |
| 24.2, 39a.2 | Gly2 | UCC | UCC | UCC | UCC | A.t. AC065824; 102745 (com) |
| 21, 25.1, 29.1, 29.2 | His | GUG | " | " | GUG | A.t. AC007383; 38526 |
| 74.1 | Ile | GAU | GAU | " | IAU | M.o. D82608; 561 |
| 8.2, 28.2a | Leu1 | UAG | " | UAG | UAG | A.t. AC011681; 66113 |
| 8.1, 28a.1 | Leu2 | GAG | " | " | IAG | O.s. U12171; 883 (com) |
| 4.1, 5.1, 6.1 | Leu3 | NAA | " | CAA | C<sup>a</sup>AA | A.t. AL096882; 9099 |
| 4.2, 4.3, 5.2, 5.3, 6.2, 6.3 | Leu4 | NAA | " | UAA | U<sup>a</sup>AA | A.t. AC069819; 72770 |
| 14 | Val1 | UAN | NAC | CAC<sup>b</sup> | CAC | A.t. AC007232; 22459 |
| 15.1, 15.2 | Val2 | GAC | GAC | AAC<sup>b</sup> | IAC | A.t. AL132965; 97564 |

<sup>a</sup> Individual tRNAs were identified by nucleotide sequence analysis, as described in the text. Numbers and letters preceding the decimal point correspond to those in Fig. 2: numbers following the decimal point refer to mobility in the third electrophoretic separation (see Fig. 3).

<sup>b</sup> Lowercase letters denote nucleosides whose identification is tentative; N, unidentified nucleoside.

<sup>c</sup> See text. I, inosine; C*, modified cytosine; U*, modified uridine.

<sup>d</sup> Two-letter abbreviations denote species name: A.t., A. thaliana; T.a., T. aestivum (wheat); M.p., M. polymorpha (liverwort); O.s., O. sativa (rice).

<sup>e</sup> Numbers following the species name are the GenBank™ accession number and the coordinate within that sequence specifying the beginning of the tRNA sequence (com = complementary strand).

<sup>f</sup> RT sequencing did not yield data for the anticodon.

<sup>g</sup> Wobble position A presumed to be converted to I post-transcriptionally (see text).

<sup>h</sup> PCR product not generated (Ala (ugN), Asp (GUC), Ile (GAU), Leu (GAG)) or was not correct (His (GUG)).

<sup>i</sup> DNA sequencing of both PCR and RT-PCR yielded evidence of only the C anticodon.

**gous Marchantia and Arabidopsis sequences.** At several other positions (A49:U65, G59, A60, G70), our sequence matches the homologous Marchantia sequence rather than the published wheat germ sequence. Position 34 is A in the Marchantia and Arabidopsis sequences and inosine in the published wheat sequence; therefore, the anticodon tentatively identified here as **GAU** by direct sequencing is very likely **IAU**.

**Leucine—**The wheat **Leu1** (anticodon UAG) is virtually identical to an **Arabidopsis** tRNA<sub>Leu<sup>i</sup></sub> sequence (Table III and Fig. 4). The **Leu2** (anticodon **GAU**) is most similar to a rice tRNA<sub>Leu<sup>i</sup></sub><sup> epid</sup> species with anticodon IAG. Although no PCR data were obtained for wheat **Leu2**, other homologous plant tRNA gene sequences have only A at position 34, with the homologous lupin tRNA<sub>Leu<sup>i</sup></sub> (39) also having an IAG anticodon.

**Wheat Leu3** (anticodon confirmed as CAA by PCR; Table III) is virtually identical to an **Arabidopsis** tRNA<sub>leu<sup>AA</sup></sub> sequence, the pair differing by only two substitutions in the variable loop. Wheat **Leu4** (anticodon UAA by PCR) is likewise highly similar to the gene sequence for an **Arabidopsis** tRNA<sub>Leu<sup>AA</sup></sub> in yeast. Two different tRNA<sub>Leu<sup>AA</sup></sub> species have either a modified U (40) or a modified C (41) in the wobble position, restricting base pairing to either UUA or UUG codons, respectively. The same situation probably also exists in mammals (42) and plants (1, 25). Judging by the results of direct sequencing of the wheat **Leu3** and **Leu4** tRNAs, the wobble nucleoside in both species also appears to be modified.

**Valine—**The wheat **Val1** sequence appeared to have a UAC anticodon on the basis of direct sequence analysis but CAC (in two independently isolated clones) by PCR amplification. With a wheat mitochondrial tRNA preparation, sequencing of an RT product provided evidence only of the C anticodon, which we therefore assign to wheat **Val1**. Homologous **Arabidopsis** sequences all have C at the wobble position. Wheat **Val2** is virtually identical over the sequenced region to a number of **Arabidopsis** tRNA<sub>Val</sub> sequences from different chromosomes. Although direct sequencing suggested a **G**AC anticodon, PCR analysis gave AAC. The corresponding lupin tRNA<sub>Val</sub> sequence has an IAC anticodon (34), which wheat **Val2** most likely has, as well.

**Determination of Import Status of Cytosolic-like Mitochondrial tRNAs—**Most of the 16 cytosolic-like tRNAs isolated from wheat mitochondria had an RRC > 0.9 (Table II), indicating that these tRNAs are present in wheat mitochondria at levels substantially exceeding those observed for cy-tRNAs that are not required by the mitochondrion for protein synthesis. Notably, the RRC for one cytosolic-like tRNA, tRNA<sub>U*AA</sub>, was significantly greater than the usual range of 1–2, which may indicate that this tRNA is not used or is not required in large amounts in the cytosol, and/or is required in mitochondria in greater relative proportion than it is in the cytosol. In a different case (cytosolic-like tRNA<sub>Leu<sup>AA</sup></sub>), the RRC was only 0.47. The latter tRNA was isolated from a major band in the two-dimensional polyacrylamide gel, and in an additional gel electrophoretic purification step (see above), it produced a band similar in intensity to that of the tRNA<sub>U*AA</sub>. Several possibilities may explain this result. (i) The oligonucleotide used in this experiment may not have been completely specific for cytosolic tRNA<sub>Leu<sup>AA</sup></sub>. However, the fact that it was successfully used for RT sequencing provides at least partial confirmation of its hybridizing target in the mitochondrial RNA fraction. (ii) The tRNA<sub>Leu<sup>AA</sup></sub>-specific oligonucleotide may have bound nonspecifically to other RNA species, particularly cytosolic RNAs. This was a potential problem for all of the slot blot hybridizations and was addressed in a control experiment using a tRNA<sub>Leu<sup>AA</sup></sub>-specific oligonucleotide having a single nucleotide mismatch with its target sequence. This oligonucleotide did not generate a visible signal within the usual time period for the slot blot hybridization experiments, although a faint signal was obtained upon much longer exposure (data not shown). (iii) The tRNA<sub>Leu<sup>AA</sup></sub> may actually constitute a greater proportion of the cytosolic RNA than it does of the mitochondrial RNA. The amount of this tRNA in mitochondria is comparable to that of other tRNAs having RRCs close to 1.0; nevertheless, the slot blot hybridization data presented here cannot unambiguously
FIG. 4. Primary sequences and potential secondary structures of wheat nDNA-encoded mt-tRNAs (see Table III), derived from chemical and reverse transcriptase sequencing data. Positions that could not be identified by direct or RT sequencing or by PCR amplification are indicated by the letter N; those tentatively identified are denoted by lowercase letters, with probable or possible dihydrouridine residues indicated by d. The filled square denotes absence of a nucleotide at that position. The sequence of the 5'-terminal region of tRNAVal was not determined. A small lowercase letter within the latter structure and beside the other structures is the corresponding nucleotide at that position in the reference DNA sequence listed in Table III. An uppercase letter beside a structure indicates that the reference sequence is RNA rather than DNA, with asterisks (*) denoting positions of post-transcriptional modification. Positions of apparent disagreement between direct sequencing and PCR analysis or positions of heterogeneity in individual PCR clones are denoted by a slash (e.g. G/A), I, inosine.
Potential codon recognition pattern in the wheat mitochondrial translation system

Codons are in uppercase letters; assigned anticodons of the corresponding tRNAs (see Table I) are in lowercase letters (i, inosine; l, lysidine; c2, modified C; u2, modified U). A minus sign (−) indicates that a tRNA corresponding to that particular codon has not been identified, whereas a number sign (#) indicates that the codon may be recognized by a tRNA that pairs with other codons in that box. Standard font denotes a native mtDNA-encoded tRNA; italicized font denotes a chloroplast-like mtDNA-encoded tRNA; bold font denotes a nDNA-encoded mt-tRNA. The deduced codon recognition pattern is based on standard wobble rules. The mtDNA-encoded tRNAs were previously characterized in Ref. 2 except for tRNAAsp, which along with the nDNA-encoded tRNAs was identified in the present study (see text).

| Codon | Phe | Ser | GGA |
|-------|-----|-----|-----|
| UUU   | gaa | UCU | Ser gga |
| UUC   | gaa | UCC | Ser gga |
| UUA   | u*aa | UCA | Ser uga |
| UUG   | c*aa | UCG | Ser uga |
| CUU   | Leu iug† | CCU | Pro # |
| CUC   | Leu iug† | CCC | Pro # |
| CUA   | Leu uag† | CCA | Pro ugg |
| CUG   | Leu uag† | CCG | Pro ugg |
| AUU   | Ile iau* | ACU | Thr |
| AUC   | Ile iau* | ACC | Thr |
| AUA   | Ile lau | ACA | Thr |
| AUG   | Met cau | ACG | Thr |
| fMet  | cau | |
| GUU   | Val iac* | GCU | Ala ige* |
| GUC   | Val iac* | GCC | Ala ige* |
| GUA   | Val iac* | GCA | Ala ugc |
| GUG   | Val cac | GCG | Ala ugc |

*Wobble nucleoside presumed to be I based on the presence of an A at this position in the corresponding gene sequence (see text).

To examine the possibility of low level expression of the wheat mt-tRNAAsp gene, a gene-specific oligonucleotide was used as a primer in RT sequencing. A clear sequence ladder was generated, with a strong stop at the position marking the mature 5′ end of the tRNA, as well as pauses within the ladder itself, diagnostic of the presence of modified nucleosides (data not shown). This makes it unlikely that the sequence ladder was produced from low levels of mtDNA in purified mitochondrial RNA preparations. The sequence for this tRNAAsp was identical to the previously published gene sequence (26) at all positions that could be discerned.

**DISCUSSION**

**Subcellular Localization of tRNAs**—From the data presented here, 14 of 16 cytosolic-like mt-tRNAs are considered to be imported into wheat mitochondria. These imported tRNAs almost entirely complement the codon recognition and amino acid specificities of the previously characterized mtDNA-encoded tRNA population. Moreover, the majority of these tRNAs constitute approximately the same proportion of either the cytosolic or mitochondrial tRNA populations, the two exceptions being tRNAAsp which represents a significantly greater (−5-fold) proportion of the mitochondrial RNA than it does of the cytosolic RNA, and tRNAVal which comprises a much smaller proportion of the mitochondrial RNA than of the cytosolic RNA. From the slot blot analysis, it is not possible to assess the import status of those nDNA-encoded tRNA species whose relative abundance in mitochondria does not significantly exceed that of a cy-tRNA that is not required for mitochondrial protein synthesis. However, even though we cannot definitively classify the tRNAAsp* species as an imported one, it is presumably required by the wheat mitochondrial translation system because no mtDNA-encoded counterpart has been found.

The mtDNA-encoded mt-tRNAAsp (detected here by RT sequencing) also appears to be present in relatively low amounts in wheat mitochondria, given that it eluded detection by direct sequencing of electrophoretically separated mt-tRNAs in the present and a previous (2) study. A similarly low level of mtDNA-encoded tRNAAsp in potato mitochondria has been noted (3). In considering how the evolutionary replacement of a mtDNA-encoded tRNA by a nDNA-encoded species might occur, we anticipate a transitional stage in which both tRNAs are functional in mitochondria, thereby allowing subsequent loss of the mitochondrial gene. When low levels of both nDNA-encoded and mtDNA-encoded species are detected in the mt-tRNA population, it is conceivable that the nDNA-encoded species is in fact required because the mtDNA-encoded tRNA is present at insufficient levels to support mitochondrial translation on its own. The case of tRNAAsp described here may represent an example of an intermediate stage in the loss of expression of a mtDNA-encoded tRNA. A similar redundancy of nDNA- and mtDNA-encoded tRNAVal species has been reported in *Marchantia* mitochondria (45).

**The Wheat Mitochondrial tRNA Population**—In view of the apparent absence of any tRNA*Leu* genes in wheat mtDNA, wheat mitochondria may well utilize the nDNA-encoded tRNA*Leu* identified here. If so, the characterized wheat mt-tRNA population would include 10 native, 6 chloroplast-like and 15 nDNA-encoded tRNAs, for a total of 31 distinct species. The potential codon recognition pattern of these tRNAs is presented in Table IV. All 61 sense codons have been identified in the collection of wheat mitochondrial protein genes sequenced to date, and there is no evidence of any departure from the standard genetic code (46). That being the case, it is evident that not all of the required wheat mt-tRNAs have been identified; in particular, (a) tRNA(s) specific for threonine remain(s) to be found.

Because the wobble pairing rules for codon/anticodon recognition have not yet been established for the plant mitochondrial translation system, it is unclear how many wheat mt-tRNA species are actually required to support protein synthesis in the organelle. In vertebrate mitochondria, expanded wobble base pairing in conjunction with a modified genetic code re-
duces the number of tRNAs required for translation to only 22–23, depending on whether the same or different tRNAsMet are used for initiation and elongation (46). In angiosperm chloroplasts, which like plant mitochondria use the standard genetic code, a minimum of 32 tRNAs is required if conventional wobble base pairing occurs (47), but only 30 tRNAs have been identified. However, Pfitzinger et al. (47) have demonstrated that chloroplast tRNAArg^{AUU} and tRNAArg^{UCU}, tRNALeu^{CAU} and tRNAPro^{CGG}, and tRNAPro^{UGG} are able to read all four codons of the respective amino acid families, apparently employing a “two out of three” base pair recognition mechanism.

It is possible that eight tRNAs having UNN anticodons could decipher the 32 codons represented by the eight four-codon families of the standard genetic code. Table IV shows that wheat mt-tRNAs of this type have been identified for five of these eight families. However, in four of these cases, tRNAs bearing either a GNN or INN anticodon have also been identified, rendering U-N wobble pairing unnecessary. This might suggest that expanded wobble pairing does not occur in wheat mitochondria, in which case a tRNAPro^{UGG} would remain to be found.

Finally, it is necessary to account for recognition of CGG (Arg) and AGA (Arg) codons, for which corresponding tRNAs have not been found. The tRNAArg^{UGG} anticodon was characterized here by both RT sequencing and PCR analysis, and an unmodified wobble position C has been confirmed by direct sequence analysis of this tRNA species. Thus, it is unlikely that tRNAArg^{UGG} would be able to decode both AGA and AGG codons. The remaining arginine codon (CGG) may be recognized by “native” tRNA genes specifying 27 distinct species (49). No mtDNA-encoded tRNAs. However, even allowing for both Gdria, liverwort mitochondria contain the largest number of the wheat mt-tRNAHis is imported. This is in contrast not only to the situation in several dicots (53, 54) but also several other graminaceous plants (55–57), where the mt-tRNAHis is a chloroplast-like, mtDNA-encoded species. Similarly, there is no tRNAPro^{UGG} gene in the mitochondrial genome of A. thaliana (19); instead, a cytosolic tRNAPro^{UGG} is imported into Arabidopsis mitochondria (43), a signal departure from other dicots. Another difference between relatively closely related plants concerns tRNAArg^{UCU} and tRNAArg^{UGA}, which are imported species in sunflower (44) but are native mt-tRNAs in potato (3) and other dicots.

In view of the number of documented differences with respect to which tRNA species are imported into mitochondria within the range of monocotyledonous and dicotyledonous plants, distinctions between these two groups are becoming increasingly blurred. Clearly, before more definitive comments can be made about the differences in the imported tRNA populations of monocot, dicot, and gymnosperm mitochondria, a larger number of plant species will have to be studied. Nevertheless, the differences demonstrated to date serve to emphasize that the plant mitochondrial translation system is quite flexible with respect to the genetic origin of the tRNAs it uses. Moreover, the import system must be able to adapt relatively rapidly over evolutionary time with regard to the particular cytosolic-like tRNAs that are brought into mitochondria to function there.

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