Aspartate and asparagine tRNA genes in wheat mitochondrial DNA: a cautionary note on the isolation of tRNA genes from plants

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ABSTRACT
We have identified genes encoding a "native" tRNA$^{Asp}$ (tmD-GTQ) and a "chloroplast-like" tRNA$^{Asn}$ (tmN-GTT) on opposite strands and 633 bp apart within a sequenced 1640 bp Rsal restriction fragment of wheat mtDNA. The tmD gene has been found previously at a different location in wheat mtDNA (P.B.M. Joyce et al. 1988 Plant Mol Biol. 11, 833-843); the duplicate copies of this gene are identical within the coding and immediate flanking regions (9 bp downstream and at least 68 bp upstream), after which obvious sequence similarity abruptly disappears. The tmN gene is identical to its homolog in maize ctDNA; continuation of sequence similarity beyond the coding region suggests that this gene originated as promiscuous ctDNA that is now part of the wheat mitochondrial genome. In the course of this work, we have encountered some unexpected similarities between tRNA gene regions from wheat mitochondria and other sources. Detailed analysis of these similarities leads us to suggest that tmN genes reportedly from petunia nuclear DNA (N. Bawnik et al. 1983 Nucleic Acids Res. 11, 1117-1122) and lupine mtDNA (B. Karpiiiska and H. Augustyniak 1988 Nucleic Acids Res. 16, 6239) are, in fact, from petunia mtDNA and lupine ctDNA, respectively, whereas a putative wheat nuclear tRNA$^{Ser}$ (tmS-TGA) gene (Z. Szwekowska-Kulińska et al. 1989 Gene 77, 163-167) is actually from wheat mtDNA. In these instances, it seems probable that the DNA samples used for cloning contained trace amounts of DNA from another sub-cellular compartment, leading to the inadvertent selection of spurious clones.

INTRODUCTION
A number of fundamental questions remain to be answered about translation and the genetic code in plant mitochondria, including how many tRNA genes are encoded by plant mitochondrial DNA (mtDNA) and how these genes are organized and expressed. We are attempting to answer these particular questions through a systematic investigation of tRNA genes in wheat mtDNA. To date, studies by us [1-7] and others [8-11] have provided evidence of at least 16 different tRNA genes in the wheat mitochondrial genome. With the exception of the initiator methionine tRNA gene [1], these tRNA genes are not in close physical linkage with each other or with non-tRNA genes, but instead are solitary. While many of the identified wheat mitochondrial tRNA genes are single-copy, several (e.g., proline [3]) are duplicated, and the members of one pair (glutamine) differ in primary sequence at a single position [4,6]. Whether both copies of such duplicate pairs are expressed is not known.

A surprising feature of some plant mitochondrial tRNA genes is their exceptionally high degree of similarity to the homologous genes in the chloroplast. Such chloroplast-like (ct-like) tRNA
genes not only appear to have been acquired directly from the chloroplast (as so-called "promiscuous" ctDNA [12]) but are also expressed in plant mitochondria (see [7] and references therein). Marechal et al. [8] originally identified a ct-like tRNA<sup>Trp</sup> gene in wheat mtDNA, and we have recently described three others, encoding cysteine (GCA), phenylalanine (GAA) and serine (GGA) isoacceptors [7]. We have also isolated and partially sequenced tRNAs corresponding to these four genes, as well as ct-like elongator methionine and asparagine tRNAs, genes for which have been reported in other plant mtDNAs [13,14].

In the present report, we describe the isolation and sequence analysis of a wheat mitochondrial tRNA<sup>Asn</sup> (trnN-GTT) gene, corresponding to the ct-like tRNA<sup>Asp</sup> we have previously identified [7]. This gene is 633 bp away from and in reverse orientation to an identical copy of a previously reported [2] tRNA<sup>Asp</sup> (trnD-GTC) gene. In the course of this study, we uncovered some unexpected similarities between two wheat mitochondrial tRNA genes (including the tRNA<sup>Asn</sup> gene described here) and three published plant tRNA gene sequences [14-16]. Our analysis leads us to question the reported subcellular origin of the clones containing these latter genes.

MATERIALS AND METHODS

Isolation of wheat mitochondrial, chloroplast and nuclear DNA

Wheat mtDNA was prepared from isolated wheat embryos by a procedure [17] involving (i) sarkosyl lysis of a highly purified mitochondrial fraction, (ii) incubation of the lysate with RNases T1 + A followed by pronase, and (iii) recovery of DNA by centrifugation in CsCl-ethidium bromide density gradients. Prior to lysis, mitochondria were freed of contaminating nuclear DNA (nDNA) by several low-speed centrifugations followed by treatment with DNase I, after which the mitochondria were banded in a discontinuous sucrose density gradient. Wheat ctDNA was isolated from light-grown wheat seedlings according to the protocol of Brookjans et al. [18], except that (i) the procedure was scaled down for 50 g of wheat leaves, (ii) three additional low speed centrifugations were carried out to exclude contaminating mitochondria and nuclei from the chloroplast preparation, and (iii) chloroplast RNA was eliminated by digestion with RNases T1 + A, rather than by passage through a Sephacryl S-1000 column. Wheat nDNA was prepared as described above for mtDNA, starting with a purified nuclear fraction freed of contaminating mitochondria and etioplasts by several low-speed centrifugations [17].

Cloning and DNA sequencing

Wheat mtDNA was hydrolyzed with HindIII and fractionated by electrophoresis in a 0.6 % low-melting-point agarose gel. Fragments from the high-molecular-weight (>9 kbp) region of the gel were recovered and ligated into pUC18, and the resulting recombinant plasmids were used to transform E. coli JM83 (see [2] for further details). Transformants were selected on the basis of their hybridization to a probe of 32P-labeled wheat mitochondrial tRNA [3]. Among the positive clones was one (p114) containing an ~9 kbp insert (HindIII fragment "c" in Fig. 1 of [2]). The tRNA hybridizing region of p114 was localized to a single ~1.6 kbp RsaI fragment. This band
was extracted from a low-melting-point agarose gel and combined with Accl-cleaved M13mp19 RF [19]; non-complementary vector ends were filled in using E. coli DNA polymerase I (Klenow fragment), and vector and insert were ligated together. Deletions of the Rsal insert were produced by the Cyclone™ system [20] for subsequent sequencing by the dideoxy chain termination method [21] using the Sequenase™ system [22]. Each nucleotide position was determined at least twice from the same or opposite strand; both strands were sequenced over the regions containing the two tRNA genes (tmD and tmN) discussed below.

Probe preparation and hybridizations
For hybridization experiments, labeled DNA probes were synthesized by allowing the sequencing reactions [21] to proceed in the absence of dideoxynucleotides. A tRNAASN gene-specific probe was produced from a template in which the first 475 nucleotides (including the tmD gene) had been deleted (see Fig. 3B), while a tRNA^Ser(TGA) gene-specific probe was generated from H-S2, a 2.5 kbp HindIII fragment containing the tmS-TGA gene [2]. The tRNA probe consisted of unfractionated wheat mitochondrial tRNAs 3'-end-labeled with [5'-^32P]pCp [23].

Southern hybridization experiments were performed using BIOTRANS nylon membranes according to the protocols provided by the vendor (ICN). All probes were heated in a boiling waterbath for 10 min and cooled on ice immediately before hybridization.

RESULTS
Sequence and organization of physically linked tmD and tmN genes
The complete sequence of a 1640 bp Rsal fragment of wheat mtDNA, containing the tRNA-hybridizing region of an ~9 kbp HindIII restriction fragment, is shown in Fig 1. Two tRNA genes are present, encoding a native tRNAASN (tmD-GTQ) and a ct-like tRNAASN (tmN-GTT). The two genes are opposite in orientation and 633 bp apart.

The tmD gene is identical in sequence to a copy of the same gene located elsewhere in the wheat mitochondrial genome [2]. Sequence similarity between the two tmD gene regions extends from the beginning of the Rsal fragment to position 168 (vertical arrow, Fig. 1), at which point obvious homology abruptly ends. Just before this point, the two sequences briefly diverge within a pyrimidine-rich stretch, immediately downstream of the tmD coding region; this stretch encompasses slightly different versions of short tandem direct repeats (overlined, Fig. 1).

The tmN gene, whose potential secondary structure is shown in Fig. 2, is identical in sequence to a maize chloroplast tmN gene [24] as well as to a ct-like tmN gene reportedly from lupine mtDNA [14] (see below). In these two cases, as well as when the comparison is made with the same regions in tobacco and liverwort (Marchantia polymorpha) ctdNAS, sequence similarity extends for a short distance into the upstream and downstream regions flanking the tmN gene itself (homologies are delineated by square brackets, Fig. 1). Southern hybridization experiments suggest that the tmN gene is a single copy gene in wheat mtDNA. The hybridization pattern (Fig. 3A) with a probe containing the tmN (but not the tmD) gene is fully consistent with the distribution.
Figure 1. Complete nucleotide sequence of a wheat mtDNA Rsal-Rsal restriction fragment (1640 bp) containing aspartate (Asp) and asparagine (Asn) tRNA genes. Positions 1-2 and 1639-1640 are inferred from restriction data. The tRNA coding regions are enclosed in solid lines and the direction of transcription is indicated by the large horizontal arrows. Both strands of the tRNA<sup>Asn</sup> gene are shown, with the tRNA-like strand in lower-case letters. Between the leftward Rsal site (i.e., the beginning of the restriction fragment) and the position of the vertical arrow, the tRNA<sup>Asp</sup> coding and flanking regions are identical to another region of wheat mtDNA containing a second copy of the same gene; the only difference is a short pyrimidine-rich stretch immediately 3′ to the coding region, which consists of slightly different short tandem direct repeats (overlined with small horizontal arrows) in the two cases. The segment delineated by parentheses ( ( ) ) shows sequence similarity with an internal portion of the <i>rpoC</i> gene in tobacco [35] and liverwort (<i>Marchantia polymorpha</i>) [36] ctDNA. Segments contained within square brackets ([ ] ) are evidently homologous (>70% sequence identity) with: (1) a region reportedly from lupine mtDNA [14]; (2) a region in maize ctDNA [24]; (3) a region in tobacco ctDNA [35]; and (4) a region in liverwort ctDNA [36]. The dashed line encloses a segment showing high sequence identity (84.3%) with a region reportedly from petunia nDNA [15].

of restriction sites within the sequenced Rsal fragment (Fig. 3B), assuming that this fragment occurs only once in the genome. We do note, however, the presence of an additional faint band of different mobility in each of the digests (solid triangles, Fig. 3). This extra band is more readily apparent in an extended exposure of a HindIII Southern blot (solid arrow, Fig. 4). Because the hybridization probe contained substantial upstream and downstream sequence in addition to the
Figure 2. Potential secondary structure of the ct-like tmN gene. Differences from the tobacco chloroplast tmN gene sequence are boxed. Note that the wheat mitochondrial tmN gene sequence is identical to the sequence of the maize chloroplast tmN gene as listed in Fig. 2 of [24], whereas Fig. 3 of the same paper omits T46, as does the sequence for the maize gene listed in Sprinzl et al. [37].

*tmN* coding region (Fig. 3B), the faint band could represent a stretch of non-coding sequence repeated elsewhere in the genome. Alternatively, a second copy of the *tmN* gene may exist, but it may not hybridize efficiently with the *tmN* probe used here because of differences in the coding and/or flanking regions. We recently encountered just such a situation during the characterization of a second glutamine tRNA gene in wheat mtDNA [6]. However, the extra band in Fig. 4 does not hybridize with a mitochondrial tRNA probe, whereas both copies of the *tmQ* gene do.

Physical linkage of the ct-like *tmN* gene with a native *tmD* gene (a duplicate copy of which resides elsewhere in wheat mtDNA [2]) provides evidence that the Rsal fragment characterized here is not derived from wheat ctDNA contaminating the mtDNA used for cloning. Further evidence supporting this conclusion comes from hybridization of the *tmN* probe to *HindIII* restriction fragments of wheat ctDNA and mtDNA (Fig. 4). As expected, the probe hybridized strongly at the appropriate position (~9 kbp) in the mtDNA lane, but very weakly at the same position in the ctDNA lane (open arrow, Fig. 4). We attribute the latter signal to slight residual contamination of the ctDNA preparation with mtDNA. The major signal in the ctDNA lane (but one still much weaker than that in the mtDNA lane) was at the position of a small (<1 kbp) *HindIII* fragment. We note that in maize ctDNA the *tmN* gene is contained within a 710 bp *HindIII* fragment [24].

A mature tRNA corresponding to the *tmN* gene has been isolated from wheat mitochondria and partially sequenced [7]. Considering that there appears to be only one such gene in wheat mtDNA (as discussed above), this would indicate that the ct-like *tmN* gene is expressed. Although our results at this point cannot formally exclude the possibility that the ct-like tRNAAsn found in wheat...
mitochondria is actually a chloroplast gene product (present either as a preparative contaminant of the wheat mitochondrial tRNA population or actively imported), data presented elsewhere [7] argue that this is not the case for three other ct-like, wheat mitochondrial tRNAs we have isolated. We conclude, therefore, that the wheat mitochondrial trnN gene belongs to the class of "recruited" chloroplast tRNA genes that now appear to function in plant mitochondria.

**Surprising sequence similarities among some plant tRNA gene regions**

In the course of comparisons between published sequences of plant tRNA gene regions and the
sequence of the wheat mitochondrial trnD/trnN region reported here, we have uncovered some rather unexpected similarities. Although the wheat mitochondrial trnN gene appears to have been derived from ctDNA, obvious sequence identity between wheat mtDNA and various ctDNAs only extends for a short distance upstream (<30 bp) and downstream (<15 bp) of the trnN coding region (Fig. 1). The same is true when the wheat mitochondrial sequence is compared with the sequence of a ct-like trnN region presumed to be from lupine mtDNA [14] (Fig. 1). On the other hand, a considerably greater extent of similarity is evident between the wheat mitochondrial sequence and the sequence of a trnN gene region reportedly derived from petunia nuclear DNA [15] (dashed line, Fig. 1). The extent and degree of these similarities is summarized in Fig. 5. Alignment of the putative petunia nuclear sequence with the wheat mitochondrial sequence (Fig. 6) convincingly shows that the two are homologous over the entire length of the published petunia sequence, with only two differences in the structural gene itself (the locations of these differences in the tRNA^Asn secondary structure are shown in Fig. 2). In fact, the putative petunia nuclear trnN gene is identical in sequence to the trnN gene of tobacco ctDNA (Fig. 5). The extent of sequence similarity between the wheat (monocotyledon) mitochondrial and petunia (dicotyledon) "nuclear" sequences is substantially greater than between either of these and the homologous region in various ctDNAs. These observations strongly suggest that the reported petunia trnN gene region [15] originates from mtDNA rather than from nDNA.
Figure 5. Comparison of a tmN gene region in wheat mtDNA (wheat mt; this report) and tobacco ctDNA (tobacco ct; [35]) with the corresponding regions encompassing putative petunia nuclear (petunia 'n'; [15]) and lupine mitochondrial (lupine 'mt'; [14]) tmN genes. Sequences have been aligned at the tmN coding region (solid rectangles), with vertical dotted lines denoting the extent of evident homology between adjacent pairs (% indicates the degree of sequence similarity). Numbering refers to corresponding coordinates in the published sequences, except that "39" and "42" refer to the identity of the nucleoside (C or U) occurring at these positions in the mature tRNA^A^ {31} (see Fig. 2). Note that the region containing the tmN gene is located within the inverted repeat in tobacco ctDNA [35].

The wheat mitochondrial and petunia "nuclear" sequences are more similar to one another than either is to the sequence of a ct-like tmN gene region reportedly from lupine (another dicotyledon) mtDNA [14]. The latter sequence, over its entire length, is homologous to that of the corresponding region in tobacco ctDNA (Fig. 7), at a very high (88.8%) level of identity. Although there are two nucleotide differences within the tmN coding region, the same substitutions are seen at the same positions in a variety of chloroplast tRNA^A^ {31} [24]. The fact that the lupine "mitochondrial" tmN gene region is more extensively similar to the corresponding region in a

Figure 6. Extent of sequence similarity between a tmN gene reportedly from petunia nDNA (pet 'n') and the homologous region in wheat mtDNA (wht mt) (complement of residues 895-618, Fig. 1; see also Fig. 5). Positional differences in the wheat sequence are listed below the petunia sequence, with deletions in one sequence relative to the other indicated by dashes (-). Small horizontal arrows denote short direct repeats. The tmN coding region is enclosed by the solid line, with the direction of transcription from left to right (large horizontal arrow). Brackets ( [ ] ) indicate the extent of evident sequence similarity with the tmN gene region of: (1) liverwort ctDNA (89.4% identity); and (2) tobacco (98.8%) and maize (96.4%) ctDNA.
Figure 7. Extent of sequence similarity between a *tmN* gene region reportedly from lupine mtDNA and the homologous region in tobacco ctDNA (see Fig. 5). Positional differences in the tobacco sequence are listed below the lupine sequence. Further details are given in the legend to Fig. 6. Brackets ([ ] ) indicate extent of evident sequence similarity with the *tmN* gene region of: (1) liverwort ctDNA; and (2) maize ctDNA.

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Figure 8. Autoradiogram showing the hybridization of $^{32}$P-labeled wheat mitochondrial tRNA to wheat mtDNA (mt), ctDNA (ct) and nDNA (n). Solid arrows mark characteristic mtDNA bands that can be seen readily in the nDNA lanes. Numbers indicate sizes (kbp) and positions of marker HindIII fragments of $\lambda$ DNA.

purified (see Materials and Methods) wheat mtDNA, ctDNA and nDNA probed with $^{32}$P-labeled wheat mitochondrial tRNAs. Although the ctDNA appears free of detectable mtDNA by this procedure (but see also Fig. 4), bands corresponding in position to characteristic mtDNA bands can be seen readily in the nDNA lanes (arrows). Slight but detectable contamination of wheat mtDNA by ctDNA has been documented elsewhere [7].

Cross-contamination is also evident when cloned DNA specific for the wheat mitochondrial $\text{tmS-TG}A$ gene is used to probe restriction digests of wheat mtDNA, ctDNA and nDNA (Fig. 9). The prominent, characteristic band present in the mtDNA lane is also readily apparent in both the ctDNA and nDNA (solid triangles) lanes.

DISCUSSION
In this report, we describe the isolation and sequence analysis of a ct-like $\text{tmN}$ gene from wheat mtDNA, and present evidence that this gene has been derived in evolution from promiscuous ctDNA that has been incorporated into the mitochondrial genome. The fact that the $\text{tmN}$ gene is physically linked to a "native" $\text{tmD}$ gene argues that the Rsal restriction fragment containing these two genes originates from wheat mtDNA, and not from contaminating wheat ctDNA. Further evidence that this is the case comes from hybridization experiments that clearly demonstrate that the
Fig. 9. Autoradiogram showing the hybridization of a wheat mitochondrial \(trnS\)-TGA gene-specific probe to wheat mtDNA (mt), ctDNA (ct) and nDNA (n). Solid triangles mark characteristic mtDNA bands that are also present in the nDNA lane. The sizes (kbp) and positions of marker \(HindIII\) fragments of \(\lambda\) DNA are indicated.

The clone containing the \(trnN\) gene is a mtDNA clone. Detection of a mature transcript corresponding to the \(trnN\) gene indicates that the gene is expressed, which characterizes it as one of the recruited ct-like tRNA genes that may be functional in wheat mitochondria [7].

In the course of this work, we have made a number of observations that call into question the reported genomic origin of several cloned and sequenced plant tRNA gene regions. In particular, the high level of sequence similarity between \(trnN\) gene regions from wheat mtDNA (this report) and petunia nDNA [15] strongly suggests that the petunia \(trnN\) gene is actually a mitochondrial, not nuclear, gene. Likewise, the pronounced sequence similarity between \(trnN\) gene regions from lupine mtDNA [14] and tobacco ctDNA makes it probable that the lupine gene represents a ctDNA clone, not a mtDNA one. These conclusions are based on the extent and degree of sequence similarity upstream and downstream of the \(trnN\) gene in pairwise comparisons (summarized in Fig. 5).

Given the widespread occurrence of chloroplast sequences in plant mtDNA [25], the recent finding of nuclear sequences in plant mtDNA [26], and the evidence of chloroplast sequences in plant nDNA [27-29], it is obviously difficult, on the basis of sequence comparisons alone, to make definitive statements about the subcellular origin of isolated plant tRNA genes. It is still possible that the putative lupine mitochondrial [14] and petunia nuclear [15] \(trnN\) gene regions are authentic examples of promiscuous ctDNA in lupine mtDNA and petunia nDNA, respectively. If so, the comparisons shown in Fig. 5-7 have two important implications: (1) If the lupine gene is indeed from mtDNA, there would have had to have been at least two independent origins of the ct-like \(trnN\) gene in plant mtDNA: one predating the divergence of monocotyledons and dicotyledons and leading to the \(trnN\) gene in wheat mtDNA (and, as we argue here, petunia mtDNA), and a second
(quite recent) one, leading to the tmN gene in lupine mtDNA. (2) If the petunia tmN gene region is from nDNA, it would have had to have originated from promiscuous ctDNA in petunia mtDNA (i.e., it could not have come directly from petunia ctDNA).

Additional data, such as the results of restriction and hybridization analysis, are therefore crucial in verifying the assumed origin of any cloned plant tRNA genes. In the absence of such information, one cannot exclude the possibility that tRNA clones have arisen from contaminating DNA from another subcellular compartment. Such appears to have been the case in the recent reported isolation [16] of a wheat nuclear tmS-TGA gene region, which we had previously characterized as a constituent of wheat mtDNA [2]. Experiments reported here demonstrate that there is detectable cross-contamination of DNAs isolated from different subcellular compartments, even when steps are taken to exclude such contamination. (We cannot, of course, completely exclude the possibility that wheat nDNA does contain a promiscuous copy of the wheat mitochondrial tmS-TGA gene region, but that this gene was not detected in the probing experiments shown in Fig. 9. As emphasized below, additional data are required to decide this issue.)

Even if contamination is at a very low level, cloning conditions may be such that the isolation of spurious clones is actually favored. This is particularly true of plant nDNA, which has a high repeat sequence content and a high content of 5-methylcytosine, both of which may contribute to poor cloning efficiencies in certain host-vector combinations [30]. The McrA and McrB systems of E. coli, which restrict DNA containing 5-methylcytosine [31], are expected to discriminate against plant nDNA clones [32], while promoting the cloning of any contaminating organellar DNAs, which apparently lack 5-methylcytosine [33,34]. Such organellar DNA clones could then be selected in hybridization screens that use as a probe bulk cellular tRNA (as in [14] and [15]), which contains small quantities of organellar species.

In the three cases of questionable assignment discussed here, only limited information was provided about method(s) of DNA isolation, assessment of the purity of isolated DNA preparations, and/or the specific host-vector combinations used in cloning. Nor were any data provided in support of the assigned subcellular origin of the tRNA clones studied. As the observations presented here suggest, such information is not only useful but indeed crucial in definitively identifying the genomic source of a plant tRNA gene.

After this manuscript had been completed, Bird et al. [38] published the sequence of a region of bean (Phaseolus vulgaris) mtDNA containing a homolog of the ct-like tmN gene we discuss here. The bean mitochondrial tmN gene region is 83% (residues 5-285) and 90% (residues 8-240) identical to the wheat mitochondrial (this report; complement of residues 747-1042, Fig. 1) and putative petunia nuclear [15] (residues 1-233) tmN gene regions, respectively. These similarities further reinforce our contention that the reported petunia nuclear tmN gene [15] is actually mitochondrial.
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