*Journal of Experimental Botany, Vol. 58, No. 15/16, pp. 4083–4093, 2007*
doi:10.1093/jxb/erm267
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

# Dual targeting of the tRNA nucleotidyltransferase in plants: not just the signal

**Serena Schmidt von Braun[1], Antonino Sabetti[2], Pamela J. Hanic-Joyce[3], Jun Gu[3], Enrico Schleiff[1] and Paul B. M. Joyce[2,3,*]**

[1] *LMU München, VW-Research group, Department of Biology I, Menzinger Str. 67, D-80638 München, Germany*

[2] *Department of Biology and Centre for Structural and Functional Genomics, Concordia University, 7141 Sherbrooke St W., Montréal, Québec, Canada H4B 1R6*

[3] *Department of Chemistry and Biochemistry, Concordia University, 7141 Sherbrooke St W., Montréal, Québec Canada H4B 1R6*

Received 14 August 2007; Revised 26 September 2007; Accepted 27 September 2007

## Abstract

**Enzymes involved in tRNA maturation are essential for cytosolic, mitochondrial, and plastid protein synthesis and are therefore localized to these different compartments of the cell. Interestingly, only one isoform of tRNA nucleotidyltransferase (responsible for adding the 3′-terminal cytidine–cytidine–adenosine to tRNAs) has been identified in plants. The present study therefore explored how signals contained on this enzyme allow it to be distributed among the different cell compartments. It is demonstrated that the N-terminal portion of the protein acts as an organellar targeting signal and that differential use of multiple in-frame start codons alters the localization of the protein. Moreover, it is shown that the mature domain has a major impact on the distribution of the protein within the cell. These data indicate that regulation of dual localization involves not only specific N-terminal signals, but also additional factors within the protein or the cell.**

Key words: Dual targeting, plant protein distribution, targeting signal, tRNA nucleotidyltransferase.

## Introduction

Plants have three compartments in which protein synthesis takes place: the cytosol, plastids, and mitochondria. Protein synthesis in each of these compartments requires a complete set of tRNAs. While the cytosolic and many mitochondrial tRNAs are encoded in the nuclear genome, other mitochondrial and all plastid tRNAs are encoded in the organellar genomes (Marechal-Drouard *et al*., 1993). The production of mature tRNAs for protein synthesis requires a number of processing and modification steps (Hopper and Phizicky, 2003) including the addition of a 3′-terminal cytidine–cytidine–adenosine (CCA) as no plant tRNA genes encode this sequence. Thus, a tRNA nucleotidyltransferase enzyme capable of adding this CCA sequence must be present in the three compartments that carry out protein synthesis. While studies in mammalian cells and *Saccharomyces cerevisiae* showed that mature and functional tRNAs are preferentially exported from the nucleus to the cytosol (Zasloff *et al*., 1982; Arts *et al*., 1998; Lipowsky *et al*., 1999), earlier experiments in yeast suggested a cytosolic role for tRNA nucleotidyltransferase in the repair of damaged CCA sequences (Rosset and Monier, 1965). Furthermore, tRNA nucleotidyltransferase has been implicated in the nuclear–cytosolic distribution of tRNAs in yeast (Feng and Hopper, 2002), suggesting that this enzyme shuttles between the nucleus and cytosol and may play a role in tRNA transport between these locations (Hopper and Phizicky, 2003). In contrast, little is known about the cellular distribution of tRNA nucleotidyltransferase in plants.

Interestingly, the characterized tRNA nucleotidyltransferases in eukaryotes are ‘sorting isozymes’, i.e. multiple isoforms of the protein can be synthesized from a single gene and distributed to different subcellular compartments. In *S. cerevisiae* (Chen *et al*., 1992; Wolfe *et al*., 1994), *Kluyveromyces lactis* (Deng *et al*., 2000), and *Candida glabrata* (Hanic-Joyce and Joyce, 2002), and

* To whom correspondence should be addressed. E-mail: joycep@alcor.concordia.ca

also proably in mammals (Reichert *et al*., 2001; Nagaike *et al*., 2001) and *Xenopus laevis* (Keady *et al*., 2002) a single gene codes for the nuclear, cytosolic, and mitochondrial forms of tRNA nucleotidyltransferase. It appears that the use of multiple in-frame start codons allows for the production of variant forms of the enzyme that contain different targeting information. Comparisons using available tRNA nucleotidyltransferase sequences identified a single gene coding for this enzyme in plants (Fig. 1). Furthermore, complementation studies in yeast using cDNAs derived from either the *Arabidopsis* (Gu, 2000; Supplementary Fig. S1 available at *JXB* online) or *Lupinus albus* genes (Shanmugam *et al*., 1996) demonstrated the biological activity of the gene products.

Based on this, it seems likely that plants employ a similar mechanism to ensure distribution of tRNA nucleotidyltransferase not only to the nucleus, cytosol, and mitochondria but also to plastids.

There is a precedent in plants for sorting isozymes involved in tRNA function. The aminoacyl-tRNA synthetases, which catalyse the addition of amino acids to their cognate tRNAs and are thereby needed in all three protein-synthesizing compartments, are frequently shared between intracellular locations, e.g. cytosol and mitochondrion or mitochondrion and plastid (Duchene *et al*., 2005). In fact, when the entire set of *Arabidopsis* genes coding for aminoacyl-tRNA synthetases was analysed, it became apparent that for no specific aminoacyl-tRNA synthetase activity were there three genes such that each gene could code for an enzyme targeted to a different location. This suggested that the targeting of this enzyme to more than one intracellular location is a consequence of a reduction in gene number following gene transfer from the original organellar genomes to the host genome (Duchene *et al*., 2005). Thus, dual localization of other enzymes involved in tRNA synthesis and maturation, and protein synthesis occurs in plants as well, although how this dual targeting is achieved remains unknown.

Sorting isozymes, especially those showing dual targeting to mitochondria and plastids, are more prevalent than might initially have been expected (Silva-Filho, 2003). It has been argued that sorting isozymes arise when an enzyme is needed to carry out functions common to multiple cellular locations or organelles (e.g. DNA replication, transcription, translation, and so on). Given that both mitochondria and plastids derive from endosymbiotic events, one could imagine that plastids, which arrived later as enslaved cyanobacteria, might simply have taken advantage of pre-existing modifications derived from the earlier endosymbiotic event leading to the mitochondria-containing host. Nevertheless, targeting still must be specific for each organelle to allow the appropriate physiological distribution of the enzymes. Moreover, dual localization is not restricted to plastids and mitochondria, and is not even limited to duality as some

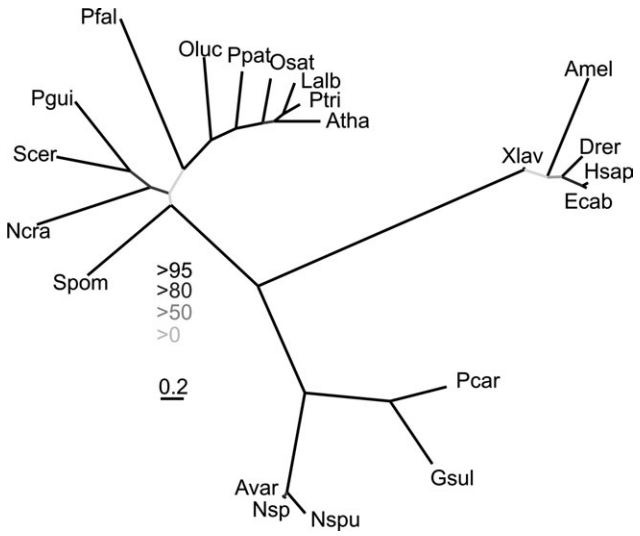



**Fig. 1.** Phylogenetic distribution of tRNA nucleotidyltransferase. The phylogeny was constructed as described. The sequence code is given in Supplementary Table S1 at *JXB* online. The colour code of the branches represents the percentage of their occurrence in 500 generated trees according to the scale indicated.

proteins may be targeted to more than two compartments (e.g. nucleus, cytosol, and mitochondria, or cytosol, plastids, and mitochondria) (Silva-Filho, 2003). Several different mechanisms to achieve this sharing have been proposed, including alternative splicing, alternative transcription/translation initiation, and the presence of 3' extensions, of ambiguous targeting sequences, and of post-translational modifications of the targeting sequence (Silva-Filho, 2003; Whelan and Schleiff, 2004; Mackenzie, 2005). Additionally, it has been proposed that cytosolic folding might act as a retention signal for further translocation (Strobel *et al*., 2002). So, it is important to ask not only if a protein is shared between multiple cellular compartments, but also how that protein is directed to more than one intracellular destination. Therefore, it is crucial to develop insights into putative regulatory mechanisms for the distribution of the protein.

In this study, the distribution of *Arabidopsis* tRNA nucleotidyltransferase (At1g22660) was analysed. A clear distribution of the protein to at least three compartments is documented: the cytosol, mitochondria, and plastids. It is shown that localization is achieved by use of at least three different translation initiation sites that produce enzymes with different targeting specificity. Unexpectedly, the analysis revealed a major influence of the mature domain of the protein, which alters the capacity for translocation toward mitochondria.

## Materials and methods

### *Phylogenetic analysis and homology modelling*

The *A. thaliana* sequence was identified using the sequence of the tRNA nucleotidyltransferase from *L. albus* (Shanmugam *et al*.,

1996). The identified amino acid sequence in *A. thaliana* was subsequently used as bait to search for additional sequences (listed in Supplementary Table S1 at *JXB* online). The sequences identified were analysed by reBLAST against the TAIR database (http://www.arabidopsis.org/). Multiple sequence alignments were produced with MAFFT v5.667 (Katoh *et al*., 2005). Bootstrap analysis was done with the PHYLIP package v3.66 (Felsenstein, 2005). Maximum-likelihood phylogenies were inferred with IQPNNI v3.1 (Minh *et al*., 2006) assuming the WAG model (Whelan and Goldman, 2001) and a gamma rate distribution.

Fold recognition of the protein encoded by At1g22660 was performed by the PHYRE server—the successor of 3DPSSM (Kelley *et al*., 2000). From the ranking of possible templates, the second hit, the crystal structure of a tRNA nucleotidyltransferase from *Aquifex aeolicus* (PDB:1VFG), was chosen as the best template. The homology model was produced automatically by the PHYRE server. Post-processing of the model was done with YASARA (http://www.yasara.org) partially remodelling the part marked in yellow in Supplementary Fig. S3 at *JXB* online, followed by an energy minimization with YASARA's Yamber3 force field (Krieger *et al*., 2004). The tRNA and ATP analogues were added to the template to illustrate better the RNA-binding site. The C-terminal ~150 amino acids of AT1G22660.1 could not be modelled safely by homology, because of a very low sequence identity to the template within this region (<<10%).

### *Mapping transcription initiation sites in* Arabidopsis

*Arabidopsis* plants (col-0) were grown for 4 weeks (16 h light/8 h dark). The above- ground parts were collected and ground in liquid nitrogen. Total RNA was isolated (from 200 mg of tissue) using the RNeasy Plant Mini Kit (Qiagen, Montréal, Canada). Transcription start sites were determined using the First Choice™ RLM-Race Kit (Ambion, Austin, TX, USA) and 10 μg of RNA. Outer and inner PCRs utilized oligonucleotide primers 1 and 2 (Table 1), respectively. Reverse transcription reactions were altered to include extension at 60 °C as follows: after the 30 min incubation with MMLV reverse transcriptase at 42 °C, a 30 min extension was performed at 60 °C with 11 U of Thermoscript™ RNase $H^-$ reverse transcriptase (Invitrogen, Burlington, Canada) in 10 μl of additional reaction buffer. PCR products were separated by agarose gel electrophoresis, purified (Bewsey *et al*., 1991), and ligated to *Eco*RV-digested pBluescript™ II $KS^+$ to which T-tails had been added using *Taq* DNA polymerase and dTTP. Plasmids were purified and DNA sequences of inserts were determined at the Centre for Structural and Functional Genomics, Concordia University. For each transcription start site, at least four independent clones were sequenced.

### *Plasmid construction*

Clone 122M21T7 containing a tRNA nucleotidyltransferase cDNA was obtained from the *Arabidopsis* expressed sequence tag (EST) sequencing project at the Arabidopsis Biological Resource Center at the Ohio State University. This served as the initial template for the PCR and was amplified first with oligonucleotides 3 and 4 (Table 1) to extend the open reading frame (ORF) to a second upstream start codon (ATG2). Subsequently this was amplified using primer 4 and primer 5 (Table 1) to introduce a further upstream start codon (ATG1). The resulting template was digested with *Nde*I and *Sal*I, cloned into pAS2, and used to generate additional products. For transformation into tobacco protoplasts, oligonucleotides 6 and 7 or 6 and 8 were used to generate long (ATG1) or short (ATG3) forms, respectively, in pBIN35S35SEmGFP after digestion with *Bcl*I and *Sal*I. For particle bombardment, the tRNA nucleotidyltransferase ORF starting from ATG1 in the plasmid p426 was amplified with oligonucleotide 9 in combination with primer 10, 11, 12, or 13 (Table 1) to generate forms of the protein starting from ATG1, ATG2, ATG3, or ATG1 (lacking ATG2), respectively, in pBIN35S35SEmGFP after digestion with *Bcl*I and *Sal*I. The region between ATG1 or ATG2 and ATG3 was amplified using the oligonucleotides 10, 11, or 12, and the 3' oligonucleotide 14, digested with *Bcl*I, and cloned into *Bam*HI-digested pBIN35S35-SEmGFP. The point mutations in the mature domain (Supplementary Fig. S2 at *JXB* online) were introduced by overlap extension site-directed mutagenesis (Ho *et al*., 1989) using the oligonucleotides 15 and 16, 17, and 18 and the appropriate terminal oligonucleotides. The altered sequence was then moved as a *Bpu*1102I fragment into the appropriate *Bpu*1102I-digested vectors. Constructs were confirmed by DNA sequence analysis (BioS&T, Montréal, Canada).

**Table 1.** *Oligonucleotides used in this study*

| Primer name | Primer sequence |
|---|---|
| Primer 1 | 5′ATCATCACTCTCTTTCCCTAGCAG3′ |
| Primer 2 | 5′ACACGAAGCTGTGTGTCAAGGTTA3′ |
| Primer 3 | 5′TACTAAAACATATGAGACTGTCTTCTCTTCCGATCAACACTCTCATAAATCTCCCAAAATCTCTCTTTCTAATTTCTCCTT3′ |
| Primer 4 | 5′ATTAGTCGACAATGTTAGTGGA3′ |
| Primer 5 | 5′CGGAATTCATATGATACTAAAAACCATGAGACTGTCTTCTC3′ |
| Primer 6 | 5′CTGCAGCTGATCAGCCTCTATCCTTTGTCGTTTAGC3′ |
| Primer 7 | 5′GTCGACTGATCATCTAGAATTCAACAATGATACTAAAAACCATG3′ |
| Primer 8 | 5′GTCGACTGATCATCTAGAATTCAACAATGACGAATGTTGGAGAGG3′ |
| Primer 9 | 5′CTGCAGCTGATCAGCCTCTATCCTTTGTCGTTTAGC3′ |
| Primer 10 | 5′GTCGACTGATCATCTAGAATTCAACAATGATACTAAAAACCATG3′ |
| Primer 11 | 5′GTCGACTGATCATCTAGAATTCAACAATGAGACTGTCTTCTCTTCC3′ |
| Primer 12 | 5′GTCGACTGATCATCTAGAATTCAACAATGACGAATGTTGGAGAGG3′ |
| Primer 13 | 5′GTCGACTGATCATCTAGAATTCAACAATGATACTAAAAACCTTGAGACTGTCTTCTC3′ |
| Primer 14 | 5′CCAACATTGATCAGCCGCCGCGTTCGTCGAGAAC3′ |
| Primer 15 | 5′CTGTATATGAGGACACTGAGGGCGAATCG3′ |
| Primer 16 | 5′CGATTCGCCCTCAGTGTCCTCATATACAG3′ |
| Primer 17 | 5′CATTTTTGAATTCTCCATGGAGATGGAGAC3′ |
| Primer 18 | 5′GTCTCCATCTCCATGGAGAATTCAAAAATG3′ |

### In vivo *analysis of protein translocation transformation*

Tobacco protoplast transformation was performed according to Matton *et al*. (1993). In brief, protoplasts were generated from leaves of 4-week-old plants, counted on a haemocytometer, and diluted to 1 million cells $ml^{-1}$. The plasmid of interest (40 μl of 1 μg $\mu l^{-1}$) was diluted with an equal volume of 2× electroporation buffer and mixed with 320 μl of protoplasts. After mixing, the samples were transferred to an electroporation cuvette (1 $cm^2$), electroporated at 225 V, 1000 μF, and placed on ice for 10 min. The electroporated protoplasts then were transferred to 3.5 ml of culture medium in a 60 mm Petri plate and incubated in the dark for 24 h. Protoplasts (20 μl) were transferred to microscope slides, covered with a coverslip, sealed, and viewed with a Leica DMIRBE confocal inverted microscope.

For the transient expression of green fluorescent protein (GFP) constructs in onion, onion bulb scales were bombarded with DNA precipitated on gold particles. For each construct, 10 μg of plasmid DNA was precipitated on 5 mg of gold particles (0.3–3 μm) in 50% glycerol with 1 M $CaCl_2$ and 15 mM spermidine under constant spinning at 4 °C for 3 min. Ethanol was added to a final concentration of 50% and the mixture was incubated for 30 min at 4 °C. The gold particles were resuspended in $H_2O$ after centrifugation, and one-tenth was used for each bombardment of onion scales. Onions were wrapped in plastic foil in the dark and incubated for 3 d at room temperature. For microscopic observation, the epidermis was peeled and stained with MitoTracker Orange (400 nM) CMTMRos (Molecular Probes, Leiden, The Netherlands) for 1 h in phosphate-buffered saline (PBS). Pictures were taken with a Leica TCS SP5 laser scanning confocal microscope (Heidelberg, Germany).

## Results and discussion

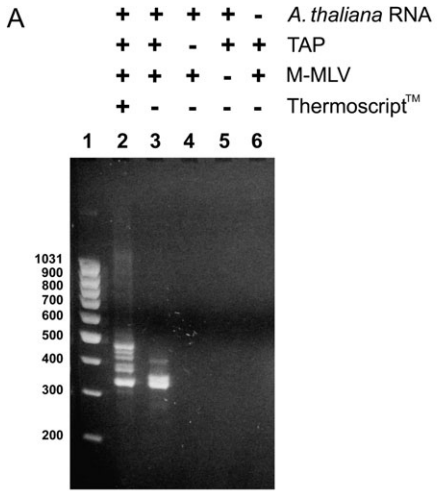
### *The gene encoding tRNA nucleotidyltransferase produces different isoforms*

Using the predicted amino acid sequence of the previously identified *L. albus* tRNA nucleotidyltransferase (Shanmugam *et al*., 1996) to carry out sequence analysis, a single gene showing significant sequence similarity was identified in the *Arabidopsis* genome. At1g22660 codes for a tRNA nucleotidyltransferase, which was identified in a proteomic analysis of mitochondria (Heazlewood *et al*., 2004). When expressed in yeast, Atlg22660 complemented a temperature-sensitive defect in yeast tRNA nucleotidyltransferase (Gu, 2000; Supplementary Fig. S1 at *JXB* online) thereby demonstrating functionality of the gene product. Using At1g22660 as bait, homologous tRNA nucleotidyltransferases were identified in fungi, metazoa, bacteria, and plants (Supplementary Table S1 at *JXB* online). Phylogenetic clustering of these sequences (Fig. 1) revealed a close relationship among the sequences found in fungi and plants, with a more distant branch including the sequences of metazoa. Interestingly, the bacterial sequences are as distant from the plant sequences as are the metazoan sequences (Fig. 1). Utilizing reverse BLAST searches, it was realized that At1g22660 does not show the highest similarity to the tRNA nucleotidyltransferase genes found in metazoa, but to the sequences of the polynucleotide adenylyltransferase family in these organisms (Supplementary Table S2 at *JXB* online). Hence, the tRNA nucleotidyltransferase analysed has close homologues in fungi, but the relationship to proteins in metazoans is less robust (Fig. 1). Therefore, the analysed tRNA nucleotidyltransferase belongs to a protein family present at least in fungi and in plants, where only one coding gene in each species can be identified.

According to the gene data bank (Tair, http://www.arabidopsis.org), two alternative splice variants exist for At1g22660, which differ near the 3' end of the gene by either having or lacking the sequence coding for two glutamines (Supplementary Fig. S2 at *JXB* online). Clone 122M21T7 was obtained, sequenced completely, and found to have the same sequence as At1g22660.1. Comparing the cDNA sequence with the gene sequence suggested that the ORF could be extended further upstream to another in-frame start codon. To map specifically where transcripts initiated, RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) and DNA sequencing were used. Four different specific transcription initiation sites were mapped by this procedure (Fig. 2A). A minimum of four independent clones was isolated for each transcription initiation site, and no additional transcription start sites were found. Some apparent heterogeneity in Fig. 2A results from size variability in the commercial adaptor used for RNA ligation prior to reverse transcription. Although all of the transcript initiation sites map upstream of the third in-frame start codon (Met69), two of these map between the codons for Met6 and Met69 and so could only produce a translation product that begins at amino acid 69. Of the two remaining transcription start sites, one is positioned to give rise to a protein starting at position 6 while the other is positioned to give rise to a translation product starting at amino acid 1 of the ORF. A similar situation exists for tRNA nucleotidyltransferase in yeast (Wolfe *et al*., 1994) and *Xenopus* (Keady *et al*., 2002) where multiple transcription start sites are positioned around three in-frame start codons. In these and other organisms (Deng *et al*., 2000; Nagaike *et al*., 2001; Reichert *et al*., 2001; Hanic-Joyce and Joyce, 2002) the use of these different start sites is thought to generate different forms of the protein lacking or containing N-terminal mitochondrial targeting information. Experiments were carried out to determine if this might also be the case in *Arabidopsis*.

### *Localization of* Arabidopsis *tRNA nucleotidyltransferase*

Based on the transcription initiation sites found and the positions of possible in-frame start codons, there are three potential translation initiation sites (Met1, Met6, and Met69) for tRNA nucleotidyltransferase (Fig. 2). The next potential in-frame start codon (Met141) is downstream of the essential DXD motive required for tRNA nucleotidyltransferase activity (Yue *et al*., 1996) and so could not be used to produce a functional protein. As a first step in defining sequences involved in the targeting of tRNA nucleotidyltransferase, an *in silico* analysis of the potential proteins generated from each start codon was carried out (Table 2). These methods did not reveal any consistent picture, even in the case of the proposed full-length transcript (see Table 2). The predicted localization ranges from cytosolic distribution to mitochondrial to Golgi to plastids, with varying degrees of confidence. Strikingly, plastid localization is favoured over mitochondrial



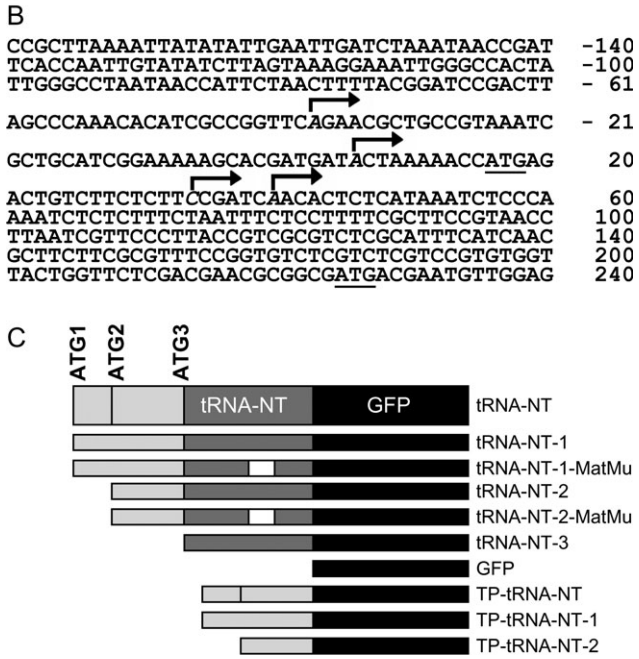







**Fig. 2.** The tRNA nucleotidyltransferase has multiple transcription initiation sites. (A) Products of RLM-RACE reactions. Size markers (lane 1) are the GeneRuler™ 100 bp DNA ladder (MBI Fermentas, Burlington, Canada). Products of inner PCRs were separated by electrophoresis in a 2% agarose gel (lane 2). Controls include reactions lacking tobacco acid pyrophosphatase (lane 4), reverse transcriptase (lane 5), or mRNA (lane 6). A modified reverse transcription procedure utilizing both M-MLV and Thermoscript™ reverse transcriptases resulted in the most efficient generation of products. (B) Positions of transcription initiation sites. The DNA sequence immediately upstream and downstream of the first three ATG codons (underlined) is shown. Each transcription initiation site is indicated by an arrow above the initiating nucleotide (italics). In (C) the constructs and the nomenclature used in this manuscript are shown. Black shows the green fluorescent protein (GFP), dark grey the protein domain, and grey the putative targeting signal. The three translation initiation sites are shown schematically. The white box in the tRNA nucleotidyltransferase region marks the point mutations introduced (Supplementary Fig. S2 at *JXB* online).

localization, although tRNA nucleotidyltransferase was identified in the proteomic analysis of the mitochondrion (Heazlewood *et al*., 2004). As the reliability of such predictions has recently been questioned (Klee and Ellis, 2005; Millar *et al*., 2006), GFP fusion constructs were used to define more precisely the localization of the gene product(s) (Fig. 2C).

When the entire coding region of tRNA nucleotidyltransferase (from ATG1) was fused to GFP (Fig. 2C, tRNA-NT) and transformed by gold bombardment into onion cells (Fig. 3), two types of transformed cells were apparent. In many cells, GFP was located only in mitochondria (Fig. 3A, left and inset), which were visualized by Mitotracker staining (not shown), but some cells showed a fluorescence in plastids as well (Fig. 3A, right and inset). Plastids can clearly be identified by their characteristic formation of stromules (Fig. 4A, right; Köhler and Hanson, 2000). These tube-like protrusions are a specific feature of plastid morphology and are thus unique identifiers (Natesan *et al*., 2005), which have been used previously to identify plastids in onions (Krause *et al*., 2005). This variability in localization was not generated by the GFP itself, as it accumulated in both the cytosol and nucleus (Fig. 3B). When this construct was electroporated into isolated tobacco protoplasts, localization to both plastids and mitochondria was observed (Fig. 3C). Here, plastid localization is determined by overlay of the GFP signal, with the autofluorescence of the organelle confirming the plastid localization in onion cells (Fig. 3A). Together these data indicate that the protein generated from the longest transcript (beginning at ATG1) contains information to target tRNA nucleotidyltransferase to mitochondria and to plastids. A question arising from this observation is whether the observed localization to mitochondria or plastids depends on the use of different translation initiation sites.

### *The N-terminus acts as a targeting signal*

Targeting of precursor proteins to plastids and mitochondria depends in most cases on an N-terminal targeting signal (Rehling *et al*., 2004; Soll and Schleiff, 2004). In good agreement with this, most of the programs utilized predicted such a signal in the *Arabidopsis* tRNA nucleotidyltransferase (Table 2). To confirm that the N-terminal region of the tRNA nucleotidyltransferase was indeed sufficient to direct the protein to both mitochondria and plastids, as seen with the full-length protein (Fig. 3A), the N-terminus itself (from amino acid 1 to 68) was fused to GFP (Fig. 2C, TP-tRNA-NT). These N-terminal 68 amino acids are indeed capable of directing GFP to both mitochondria and plastids (Fig. 4A). Mitochondria were identified by Mitotracker staining exemplarily shown in Fig. 4B, whereas plastids were identified by their characteristic stromule formation (Köhler and Hanson,

**Table 2.** *Predicted localization of the three putative At1g22660 translation products*

The putative localization of the proteins encoded by the three different transcripts was analysed by different web-based programs. Listed is the name of each program, its citation, the ATG from which the sequence was translated, putative mitochondrial signals, putative plastid signals, putative localization distinct from mitochondria or plastids, and additional information to judge the quality of the prediction.

| Program | Reference | Result | | | | Information |
|---|---|---|---|---|---|---|
| Predict NLS | Cokol *et al*. (2000) | ATG1 | | | NLS | Amino acids 597–603 |
| NetNES | La Cour *et al*. (2004) | | | | NES | Amino acids 89–98 |
| Subnuclear | Lei and Dai (2005) | | | | NUO | |
| PSORT Prediction | Nakai and Horton (1999) | ATG1 | mTS | | | Mitochondrial matrix; Score 0.512 |
| | | ATG2 | mTS | | | Mitochondrial matrix; Score 0.512 |
| | | ATG3 | | | Cyt | Score 0.450 |
| WoLF PSORT | Horton *et al*. (2007) | ATG1 | | cTP | | Score 11 |
| | | ATG2 | | cTP | | Score 13 |
| | | ATG3 | | | Cyt | Score 7 |
| ProtComp 6.0 | Klee and Ellis (2005) | ATG1 | | cTP | | Score 2.20 |
| | | ATG2 | | cTP | | Score 2.76 |
| | | ATG3 | | | Cyt | Score 1.30 |
| SherLoc | Shatkay *et al*. (2007) | ATG1 | mTS | | | Score 0.79 |
| | | ATG2 | mTS | | | Score 0.49 |
| | | ATG3 | | | Cyt | Score 0.82 |
| Plant-Ploc | Chou and Shen (2007) | ATG1 | | | Cyt | – |
| | | ATG2 | | | Cyt | – |
| | | ATG3 | | | Cyt | – |
| BaCelLo | Pierleoni *et al*. (2006) | ATG1 | | cTP | | – |
| | | ATG2 | | cTP | | – |
| | | ATG3 | | | NLS | – |
| pTARGET | Guda and Subramaniam (2005) | ATG1 | | | Golgi | Confidence, 87.6% |
| | | ATG2 | | | Golgi | Confidence, 87.6% |
| | | ATG3 | | | Cyt | Confidence, 87.6% |
| TargetP (ChloroP) | Emanuelsson *et al*. (1999, 2007) | ATG1 | | cTP | | Score: 0.749, rank 2; cTP length 50aa |
| | | ATG2 | | cTP | | Score: 0.785, rank 2; cTP length 45aa |
| | | ATG3 | | | Other | Score: 0.901, rank 2 |

cTP, chloroplast targeting signal; Cyt, predicted cytosolic occurrence; Golgi, targeting to the Golgi network; mTP, mitochondrial targeting signal; NES, nuclear export signal; NLS, nuclear localization signal; NUO, localization in the nucleolus.

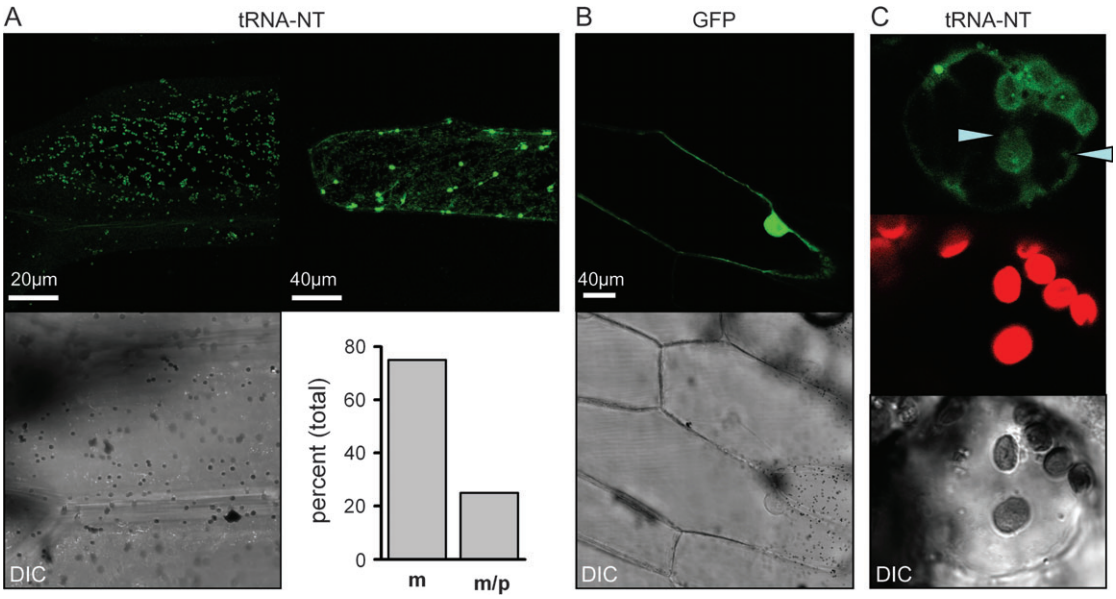



**Fig. 3.** Protein distribution *in vivo* is dependent on the targeting signal. The tRNA nucleotidyltransferase fused to GFP (A, C) or GFP itself (B) was expressed in onion cells (A, B) or tobacco protoplasts (C). GFP fluorescence (A, B, and C top) or chlorophyll autofluorescence (C, middle) was visualized by confocal microscopy. For orientation, the differential interference contrast (DIC) images are also shown (A, B, and C bottom). Mitochondria were assigned by Mitotracker staining as shown in Figs 4 and 5. Representative images are shown in A (left), B, and C. In A (right) is an example of one of the few cells seen with dual localization. Below this are the statistics indicating the relative numbers of cells showing mitochondrial (m) or dual mitochondrial and plastid (m/p) targeting. The arrows in C point to mitochondria. The white bar indicates the magnification.

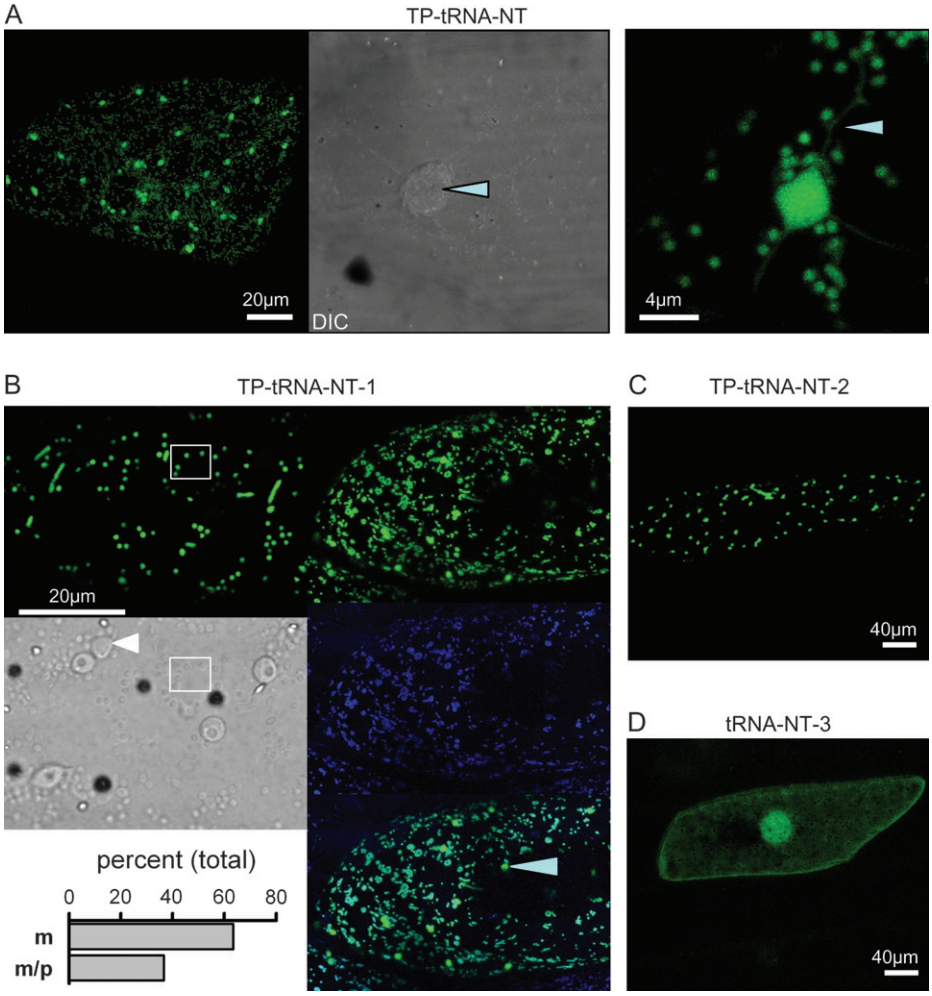



**Fig. 4.** The N-terminus of the tRNA nucleotidyltransferase acts as a targeting signal *in vivo*. The first 68 amino acids starting at the first ATG (A), the first 68 amino acids starting at the first ATG with the methionine at position 6 converted to leucine (B), the first 63 amino acids starting at the second ATG (C), or the mature domain (D) of tRNA nucleotidyltransferase starting at amino acid 69 (counting from the first ATG) fused to GFP were expressed in onion cells and GFP fluorescence analysed by confocal fluorescence microscopy. In A, the arrow in the differential interphase contrast (DIC) image points to the nucleus; the arrow in the fluorescence image (right) points to stromules. In B, the left panel shows the representative distribution of fluorescence in cells with exclusive mitochondrial localization. The box shows three mitochondria, which can be clearly seen in the DIC image. The arrow in the DIC image points toward a plastid that shows no GFP fluorescence. The inset below shows the ratio of cells showing mitochondrial (m) or dual (m/p) localization. The right panel shows an example of the cells with dual localization. Top: the GFP fluorescence. Middle: Mitotracker staining. Bottom: the overlay image. The arrow indicates a plastid that shows GFP fluorescence. The white bar indicates the magnification.

2000; Krause *et al*., 2005; Natesan *et al*., 2005). Interestingly, using this construct, no cells showing only mitochondrial localization were seen. The same result was observed in tobacco protoplasts (data not shown).

Although the constructs were made to optimize expression from the first in-frame start codon (Joshi *et al*., 1997), it is possible that protein synthesis also starts from the second in-frame ATG 15 nucleotides downstream. In fact, for some other plant proteins with dual localization, for example protoporphyrinogen oxidase (Watanabe *et al*., 2001) and THI1 (Chabregas *et al*., 2003), two in-frame start codons on the same mRNA are used to code for the chloroplast and mitochondrial forms of the protein. To investigate the possibility that different isoforms, targeted to different organelles, could be produced by using the different translation initiation sites in a single message, an A to T substitution was introduced to convert the second ATG codon (Met6) to a TTG leucine codon. When the distribution of GFP fused to these 68 amino acids was determined (Fig. 2C, TP-tRNA-NT-1), localization to mitochondria was predominantly obtained (Fig. 4B), although in approximately one-third of the cells a dual localization to mitochondria and plastids was obtained (Fig. 4B, left). In contrast, when the first ATG was removed such that translation could only initiate at the second ATG (Fig. 2C, TP-tRNA-NT-2), the encoded signal directed GFP exclusively to plastids (Fig. 4C). This indeed suggests that the different transcripts code for

proteins with different targeting signals. It appears that the 'full-length' signal is capable of targeting to both mitochondria and plastids, whereas the 'short' signal (lacking the five N-terminal amino acids) exclusively targets the protein to plastids. This is in contrast to the examples cited above where the longer form of the proteins was targeted to the plastids while the shorter forms were directed to the mitochondria. When the mature domain of the tRNA nucleotidyltransferase—lacking the 68 N-terminal amino acids—was fused to GFP, the fusion protein (Fig. 2C, tRNA-NT-3) remains in the cytosol and in the nucleus and is not directed to mitochondria or plastids (Fig. 4D). However, the ability of GFP alone to accumulate in the nucleus (Fig. 3B) compromises any conclusions about a putative nuclear localization of the tRNA nucleotidyltransferase, which also was predicted by the *in silico* analysis (Table 2).

### *The mature domain of the tRNA nucleotidyltransferase influences the efficiency of the targeting signal*

So far it has been possible to demonstrate that the N-terminal region contains the information necessary and sufficient to direct GFP to both mitochondria and chloroplasts. However, the picture obtained in comparing GFP fused to the entire protein (Fig. 3A) or the targeting sequence alone (Fig. 4A) differs with respect to the intensity of plastid targeting, i.e. fusing the whole protein to GFP decreases the signal seen in the plastids as compared with that seen when the targeting signal alone is fused to GFP. This is similar to the change in distribution seen when the second methionine of the targeting signal alone is replaced by leucine (compare Figs 3A and 4B). So although the N-terminal targeting signal is sufficient for organelle targeting, the role the mature domain has in the targeting of the protein was not known. Does the presence of the mature domain change the distribution of protein being synthesized from the first or second ATGs, or does the mature domain itself alter the targeting of the protein? To address this question, the distribution of the entire protein fused to GFP when the second methionine had been converted to leucine was first analysed (Fig. 2C, tRNA-NT-1). This protein is exclusively localized to mitochondria (Fig. 5A). Not even a single cell with localization to plastids was found. Hence, it appears that the mature domain enforces by some unknown means a specificity of targeting toward mitochondria, and that the second ATG is indeed required for dual distribution. To support this interpretation, the distribution of tRNA nucleotidyltransferase starting at the second methionine was analysed (Fig. 2C, tRNA-NT-2). This construct is localized to both plastids and mitochondria (Fig. 5B) in contrast to the targeting signal (starting from ATG2) alone fused to GFP, which was found only in plastids (Fig. 4C). Hence, the mature domain of the protein does play a role in defining its own final localization, i.e. the N-terminal targeting signal is not the only factor that influences the localization of tRNA nucleotidyltransferase.

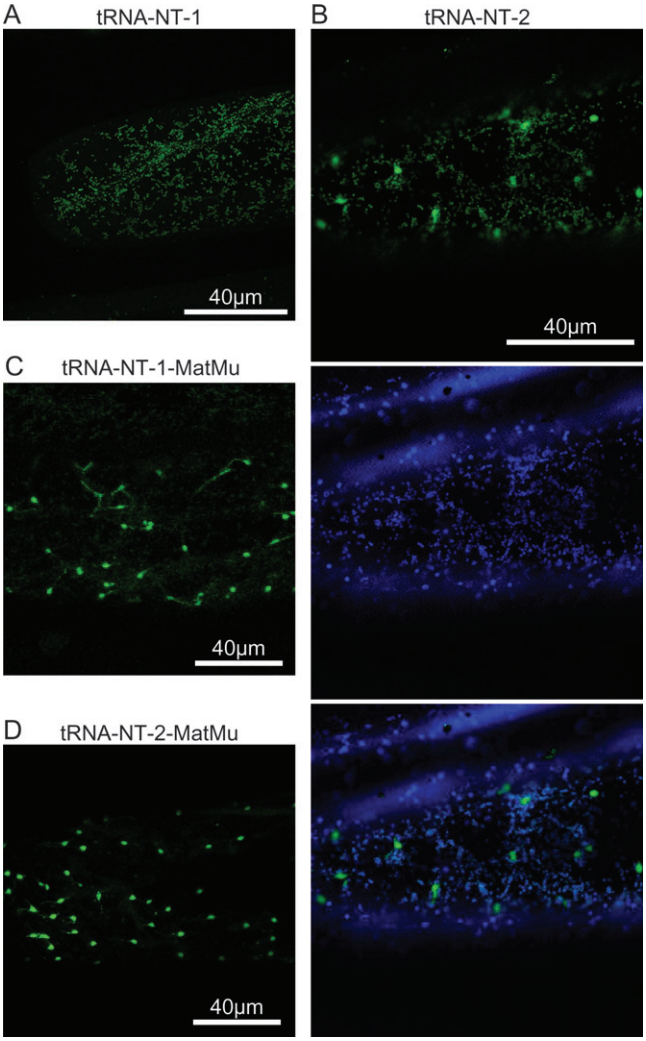



**Fig. 5.** The mature domain of tRNA nucleotidyltransferase influences the targeting signal activity. The tRNA nucleotidyltransferase starting at the first ATG with the methionine at position 6 changed to leucine (A), the tRNA nucleotidyltransferase starting at the second ATG (B), the tRNA nucleotidyltransferase starting at the first ATG with the methionine at position 6 changed to leucine, and mutations in the surface-exposed charges (MatMu, Supplementary Figs S2 and S3 at *JXB* online; C), or the tRNA nucleotidyltransferase starting at the second ATG with mutations in the surface-exposed charges (D) were fused to GFP. The constructs were expressed in onion cells and GFP fluorescence analysed by confocal fluorescence microscopy. The white bar indicates the magnification.

Although it is possible that the length of the targeting signal in the targeting signal–GFP fusion proteins was underestimated, and as such incorrect localization of the constructs was being obtained, this seems unlikely as the constructs contained the 68 N-terminal amino acids of the protein and the transit sequence was predicted to be a maximum of 50 amino acids long (Table 2,

TargetP/ChloroP). However, to exclude this possibility and to confirm the influence of the mature domain, the distribution of the tRNA nucleotidyltransferase–GFP protein with seven charged amino acids altered in the mature domain was analysed (Supplementary Fig. S3 at *JXB* online; MatMu). These amino acids are expected to be exposed to the surface as predicted by homology modelling (Supplementary Fig. S3 at *JXB* online) and show a similar signature to a bipartite-type nuclear localization signal (Robbins *et al*., 1991), which suggests that it might be an interaction site for cytosolic factors. When this mutation was introduced, the distribution of the protein starting at the first methionine (Fig. 2C, tRNA-NT-1-MatMu) was reversed compared with that found for the protein with the wild-type mature domain. An almost exclusive localization to plastids (Fig. 5C) was now obtained instead of the exclusive mitochondrial localization seen before (Fig. 5A). The same shift toward plastid targeting was obtained when the first ATG was removed and only the truncated targeting sequence was present in the fusion protein (Fig. 2C, tRNA-NT-2-MatMu; Fig. 5D). These data indicate that the N-terminal targeting signal alone is not sufficient to define the precise localization of tRNA nucleotidyltransferase. They indicate that additional regions of the protein are also involved in modulating the localization of this protein, perhaps through the involvement of additional cytosolic factors that interact not only with the N-terminal targeting sequence but also with the rest of the tRNA nucleotidyltransferase protein.

**Table 3.** *Localization of the specific GFP fusion proteins used*

This table summarizes the data shown in Figs 3–5. Each construct name is according to Fig. 2. An X in any box indicates that cells showing only that localization were observed.

| Construct name | Localization | | | |
|---|---|---|---|---|
| | Cyt | Mit | Pl | Mit and Pl |
| GFP | X | | | |
| TP-tRNA-NT | | | | X |
| TP-tRNA-NT-1 | | X | | X |
| TP-tRNA-NT-2 | | | X | |
| tRNA-NT | | X | | X |
| tRNA-NT-1 | | X | | |
| tRNA-NT-2 | | | | X |
| tRNA-NT-3 | X | | | |
| tRNA-NT-1-MatMu | | | X | |
| tRNA-NT-2-MatMu | | | X | |

Mit, mitochondrial localization; Mit and Pl, co-localization to mitochondria and plastids; Pl, plastid localization; Cyt, cytosolic distribution.

## Conclusion

In this study, it is demonstrated that tRNA nucleotidyltransferase is localized to at least three compartments in *Arabidopsis*, namely plastids, mitochondria, and the cytosol (Figs. 3, 4, summarized in Table 3), and that the distribution of this protein is controlled, in part, by the use of three different translation initiation sites (Fig. 2) present or absent in multiple transcripts. As nuclear localization of GFP alone compromises the determination of nuclear localization of tRNA nucleotidyltransferase, the localization of the enzyme to this fourth compartment of the cell cannot be excluded. Organellar targeting is dependent on the N-terminal region of the protein which contains a targeting signal as determined by both *in silico* (Table 2) and *in vivo* analysis (Fig. 4). The preferential targeting of the shorter N-terminal signal to plastids and the targeting of the longer N-terminal signal to both organelles (Fig. 4) parallels the observation for the dual-targeted glutathione reductase (Rudhe *et al*., 2002*a*). However, targeting is not defined simply by the N-terminal sequence as alteration of residues in the mature domain of the protein influenced targeting. This result implicates cytosolic factors in the targeting process rather than a simple effect conferred by altered enzyme folding, especially because current models suggest that precursor proteins are kept in an unfolded conformation prior to translocation across the membranes of the target organelle (Soll and Schleiff, 2004; Whelan and Schleiff, 2004). Additionally, even though the possibility that the changes that have been made alter the conformation of the protein affecting its localization cannot be excluded to its final extent, the modelling suggests that the altered amino acid residues are on the exterior of the protein and do not have any major interactions with other regions of the protein. Moreover, the changes that have been made, replacing positively charged lysine residues with negatively charged glutamate residues (and one arginine with methionine), should maintain the overall hydrophilic character of this region of the protein and limit changes in folding. Furthermore, even if the precursor protein is able to acquire a folded conformation in the cytosol, altered folding of proteins has been found to result in an import-incompetent state (Strobel *et al*., 2002). In contrast, the altered proteins retained import competence, but shifted from mitochondrial to plastid targeting (Fig. 5). Considering that mistargeting to chloroplasts has never been observed, at least not in *in vitro* import experiments (Rudhe *et al*., 2002*b*), a modulation of translocation efficiency by cytosolic factors would explain the findings using the mutant proteins as well as the different plastid targeting efficiency of tRNA nucleotidyltransferase seen in tobacco protoplasts as compared with onion cells (Fig. 3). In conclusion, it is shown that tRNA nucleotidyltransferase exhibits multiple localizations in plants: plastids, mitochondria, and cytosol. It remains to be explored whether tRNA nucleotidyltransferase is also present in the nucleus and whether cytosolic factors achieve the modification of translocation efficiency.

## Supplementary material

Supplementary material is available at *JXB* online.

**Table S1.** Species names, name codes, and sequence accession numbers for the sequences used in phylogenetic analysis.

**Table S2.** Identification of protein-coding regions by database similarity search using the sequences of the identified homologues.

**Fig. S1.** Complementation of a temperature-sensitive defect in the *S. cerevisiae CCA1* gene coding for tRNA nucleotidyltransferase by its *Arabidopsis* homologue.

**Fig. S2.** Comparison of the proteins encoded by splice variants of At1g22660.

**Fig. S3.** Homology model of AT1G22660.1 based on the structure of a tRNA nucleotidyltransferase from *Aquifex aeolicus* (PDB:1VFG).

**Fig. S4.** The distribution of GFP-tagged tRNA-NT, TP-tRNA-NT, and TP-tRNA-NT-2.

## Acknowledgements

We especially thank Oliver Mirus (LMU Munich, Germany) for his support in homology modelling, and Ina Rohwedder (LMU Munich, Germany) for her technical assistance. We also thank Alexandre Joyeux and Normand Brisson (Université de Montréal) for plasmids and assistance with tobacco protoplast transformation, and Krystel Kanaan for the complementation assay. This work was supported by grants to ES from the Volkswagenstiftung and to PJ from the Natural Sciences and Engineering Research Council of Canada.

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