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The $tRNA^{Tyr}$ multigene family of *Triticum aestivum*: genome organization, sequence analyses and maturation of intron-containing pre-tRNAs in wheat germ extract

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Abstract **Southern analysis of *Triticum* DNA has revealed that nuclear $tRNA^{Tyr}$ genes are dispersed at a minimum of 16 loci in the genome. We have isolated six independent $tRNA^{Tyr}$ genes from a *Triticum aestivum* library in addition to three known members of the *Triticum* $tRNA^{Tyr}$ family. Four of the sequenced $tRNA^{Tyr}$ genes code for *Triticum* $tRNA_1^{Tyr}$ and two code for $tRNA_2^{Tyr}$. Three genes encode $tRNAs^{Tyr}$ which carry one or two nucleotide substitutions as compared to the conventional genes. The nine *Triticum* $tRNA^{Tyr}$ genes possess highly conserved intron sequences ranging in size from 12 to 14 nucleotides. A common secondary intron structure with the 5′ and 3′ splice site loops separated by five base pairs can be formed by all pre-$tRNAs^{Tyr}$ which are efficiently spliced in the homologous wheat germ extract.**

Key words: Genome organization; In vitro transcription; In vitro splicing; *Triticum*; Wheat germ extract; Tyrosine tRNA genes

1. Introduction

Intron-containing tRNA genes have been found in the nuclear genomes of lower and higher eukaryotes and include quite different tRNA isoacceptors. In the yeast *Saccharomyces cerevisiae* introns occur in tRNA genes belonging to 10 different families [1–3]. Until a few years ago, intron-containing tRNA genes in higher eukaryotes were thought to be an exception since they had been detected only in tRNA genes coding for $tRNA^{Tyr}$ [4–8] and $tRNA^{Leu}$ [8,9]. But recently an intron-containing $tRNA^{Arg}$ (TCT) gene has been identified within a large cluster of human tRNA genes [10]. In addition mollusk genes encoding $tRNA^{Lys}$ (TTT) were shown to contain intervening sequences [11].

We have concentrated our efforts on the isolation and characterization of intron-containing $tRNA^{Tyr}$ genes in higher plants [12–15]. A special feature of this tRNA family is the ubiquitous presence of introns in the corresponding genes of all eukaryotic organisms studied until now. It was shown by us and other groups that the intron is a prerequisite for pseudouridine (Ψ_{35}) biosynthesis in the GΨA anticodon of mature $tRNA^{Tyr}$ [16–18] and that the Ψ_{35} synthase requires in addition a consensus sequence in the anticodon loop [19]. Furthermore we have established a wheat germ extract for efficient and faithful pre-tRNA processing and splicing using an intron-containing *Nicotiana* $tRNA^{Tyr}$ precursor as a substrate [20]. Unexpectedly we later found that the splicing endonuclease from wheat germ exhibits a strict requirement for homologous pre-tRNAs in contrast to the well-studied yeast enzyme which accepts intron-containing tRNAs from a variety of different organisms including humans and plants [21]. In order to determine whether plant introns display any common sequences or structural features we have eventually isolated 13 nuclear $tRNA^{Tyr}$ genes from different gene loci of *Nicotiana rustica* [13]. As expected, all *Nicotiana* $tRNA^{Tyr}$ genes contained intervening sequences but they differed considerably in length and sequence. To gain further insight into the specific demands manifested by wheat germ splicing endonuclease we decided to study $tRNA^{Tyr}$ genes from the homologous organism, *Triticum aestivum*.

Here we show that wheat nuclear $tRNA^{Tyr}$ genes are mainly organized as a dispersed family consisting of at least 16 members. Six of these $tRNA^{Tyr}$ genes were isolated and sequenced, in addition to three genes previously described [15,22]. Furthermore their expression was studied in HeLa cell nuclear and wheat germ extract.

2. Materials and methods

2.1. Enzymes and reagents

Restriction endonucleases, T4 DNA ligase, calf intestinal alkaline phosphatase and polynucleotide kinase were purchased from Boehringer, Mannheim. The sequenase kit from USB was used for sequencing reactions. Radiochemicals were obtained from Hartmann Analytic, Braunschweig. Nylon membrane Biodyne B from Pall, Dreieich was used for Southern blots. Untreated wheat germ was a gift from SynPharma GmbH, Augsburg.

2.2. Bacterial strains and plasmids

Escherichia coli JM109 was used as a host for propagation of plasmid DNA. The recombinant plasmids pTtY1, pTtY2 and pTtY3 carrying $tRNA^{Tyr}$ genes from different loci of the *Triticum aestivum* genome subcloned into the pUC19 vector have been described elsewhere [15,22].

2.3. Isolation of wheat genomic DNA

High molecular weight DNA was isolated from leaves of 7 day old *Triticum aestivum* sprouts. Frozen leaves were ground in a mortar under liquid nitrogen. The dry powder was dispersed in lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM Na_2EDTA, 5 mM dithiotreitol) to which 0.5% SDS and 5 mg/ml proteinase K had been added and the mixture was incubated for 1 h at 50°C followed by two extractions with chloroform/octanol (24:1). The aqueous phase was recovered and the DNA was further purified by CsCl-ethidium bromide centrifugation.

2.4. Synthesis and labelling of oligodeoxyribonucleotides

Oligonucleotides were synthesized with the Gene Assembler Plus from Pharmacia LKB. They were end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified with Qiagen tip 5 columns

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EMBL accession numbers: X71947–X71950 (pTtY4 to pTtY7) X76914 and X76915 (pTtY8 and pTtY9).

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(Diagen, Düsseldorf). Screening of a recombinant phage library was performed with a 20-mer oligonucleotide (5′-CTACAGTCCTCCGCTCTACC-3′, probe 1) complementary to nucleotides 18–37 of $tRNA^{Tyr}$ from tobacco [23]. The following two oligonucleotides were used as sequencing primers: (i) 5′-CCGACCTTAGCTCAGTTGGT-3′ (identical with nucleotides 1–20 of $tRNA^{Tyr}$, probe 2) and (ii) 5′-CCGGATTCGAACCAGTGACC-3′ (complementary to nucleotides 45–62 of $tRNA^{Tyr}$, probe 3).

2.5. Isolation of genomic clones

A *Triticum aestivum*, var. Tam 107 Hard red winter genomic library from 13 day old seedlings in the lambda phage EMBL-3 vector was received from CLONTECH Laboratories, Heidelberg. Screening of the phage library was performed with $tRNA^{Tyr}$-specific probe 1.

2.6. DNA sequencing

Direct sequencing of plasmid DNAs was carried out according to a modified dideoxy chain termination method using T7 DNA polymerase.

2.7. In vitro transcription in HeLa cell nuclear extract

Nuclear extract was prepared from HeLa S3 cells according to Dignam et al. [24]. Transcription assays and the elution of precursors from preparative gels were performed as described by Stange and Beier [20].

2.8. In vitro processing and splicing of pre-tRNAs in wheat germ extract

Cell-free wheat germ S23 extract was prepared from wheat embryos according to Stange and Beier [20]. In vitro processing and splicing of tRNA precursors was performed in a total volume of 30 µl, containing 6 µl S23 extract (25 mg protein/ml), 20 mM Tris-HCl, pH 7.4, 100 mM potassium acetate, 6 mM magnesium acetate, 80 µM spermine, 10 mM creatine phosphate, 0.8% Triton X-100, 1 mM ATP, 0.1 mM CTP and $\sim 8 \times 10^3$ cpm precursor tRNA.

3. Results and discussion

3.1. Organization of $tRNA^{Tyr}$ genes in Triticum nuclear DNA

tRNA genes in eukaryotes exist as multigene families which are either distributed randomly throughout the genome or are clustered at single chromosomal sites. We have studied the genome organization of $tRNA^{Tyr}$ genes in a variety of organisms and have found both types of tRNA gene arrangement. In the human and *Nicotiana* genomes, $tRNA^{Tyr}$ genes are mainly organized as a dispersed multigene family consisting of at least 12–15 members [13,17]. A unique organizational pattern has been detected for the $tRNA^{Tyr}$ genes in the nuclear genome of *Arabidopsis thaliana*. One $tRNA^{Ser}$ (AGA) gene and two $tRNA^{Tyr}$ (GTA) genes occur in tandem arrangement on a 1.5 kb unit which is amplified about 20-fold at a single chromosomal site [14].

We used a 5′-labelled $tRNA^{Tyr}$-specific oligonucleotide for hybridization to *Eco*RI-digested DNA from *Triticum aestivum*. Southern blot analysis revealed the existence of at least 16 hybridizing DNA fragments ranging in size from 0.65 to 6.7 kb (Fig. 1A). In parallel we repeated Southern analysis of *Eco*RI-cleaved DNA from *Nicotiana rustica* (Fig. 1B). The hybridization pattern of *Triticum* DNA clearly indicates that *Triticum* $tRNA^{Tyr}$ genes are mainly organized as a dispersed multigene family, thus resembling the $tRNA^{Tyr}$ and $tRNA^{Ser}$ gene organization in the *Nicotiana* genome [13,25]. The strong hybridization signal displayed by the 1.5 kb fragment varied in intensity in different Southern blots and may indicate that tDNA-carrying restriction fragments of approximately the same size were not resolved in the gel. As we will point out later we did not find any evidence for the existence of clustered $tRNA^{Tyr}$ genes.

3.2. Isolation and nucleotide sequences of six $tRNA^{Tyr}$ genes from Triticum aestivum

About 6×10^5 recombinant phages from a *Triticum aestivum* genomic library in the lambda EMBL3 vector were screened for $tRNA^{Tyr}$ genes with ^{32}P-labelled $tRNA^{Tyr}$-specific probe 1. Six independent recombinant phages were identified as positive clones in addition to the three clones, i.e., pTtY1 to pTtY3, described earlier [15,22]. The individual hybridizing *Sal*I fragments ranged in size from 1.25 to 7.8 kb (Table 1) and most of them were subsequently cleaved with appropriate restriction endonucleases and were then cloned into pUC19 DNA. Cleavage of all clones with a variety of other restriction enzymes did not reveal the presence of more than one $tRNA^{Tyr}$ gene on the individual *Sal*I fragments with the exception of the 5.5 kb fragment. Digestion of the latter with *Rsa*I had previously shown the existence of two $tRNA^{Tyr}$ genes on the 5.5 kb *Sal*I fragment [15]. In the meantime we

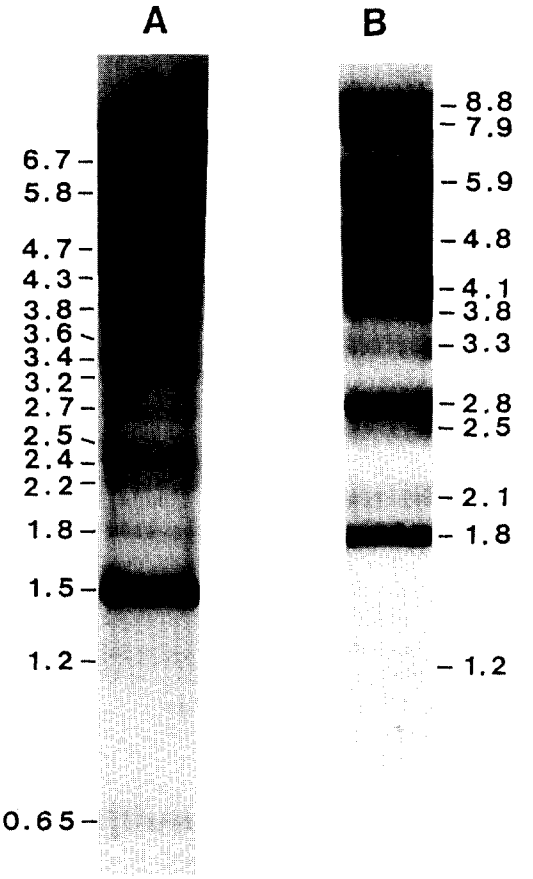

Fig. 1. Comparison of Southern hybridization analyses of genomic DNA from *Triticum aestivum* and *Nicotiana rustica*. 30 µg of *Triticum* (A) and *Nicotiana* (B) DNA were digested with 15 U *Eco*RI/µg DNA for 6 h at 37°C and subjected to electrophoresis on a 0.8% agarose gel. *Hpa*I-digested T7 DNA fragments were run as markers in adjacent lanes. Prehybridization of Southern blots was in 6×SSC, 10×Denhardt's solution, 0.01 M phosphate buffer pH 6.8, 10 mM EDTA, 1 mM ATP, 0.05% SDS, 0.1 mg/ml denatured DNA from herring sperm at 40°C for 6 h. Hybridization was at 40°C for 18 h in the same buffer with 10^6 cpm of a 5′-labelled $tRNA^{Tyr}$-specific oligonucleotide (probe 1) per ml. After hybridization the filters were washed three times with 6×SSC, 0.05% SDS at room temperature, once at 40°C for 30 min and once at 59°C for 7 min. The numbers at the left and right indicate the length of the hybridizing *Eco*RI fragments (kb).

Table 1
Characterization of the cloned genomic *Triticum* tDNA^{Tyr} fragments (underlined) contained in pTtY1 to pTtY9

Restriction enzyme	TtY1	TtY2	TtY3	TtY4	TtY5	TtY6	TtY7	TtY8	TtY9
*Sal*I	5.5 kb	5.5 kb	11.5 kb	2.2 kb	4.9 kb	6.0 kb	7.8 kb	1.25 kb	4.3 kb
*Rsa*I[a]	0.83 kb	1.3 kb							
*Pst*I			1.3 kb						
*Eco*RI					0.5 kb				
*Cfo*I						1.5 kb	1.15 kb		1.3 kb

[a]The two *Rsa*I subclones of 0.83 and 1.3 kb are contained in the same 5.5 kb *Sal*I fragment [15].

have elucidated almost the whole nucleotide sequence of this fragment and have established that the two tRNA^{Tyr} genes are not located on a 1.5 kb *Eco*RI fragment and that the distance between these two genes amounts to about 1550 bp (C. Schüll and H. Beier, unpublished).

We have sequenced the coding region of tRNA^{Tyr} genes contained in pTtY4 to pTtY9 and at least 50 nucleotides of their 5′- and 3′-flanking regions, respectively. The 0.5 kb *Eco*RI fragment of pTtY5 was completely sequenced. A comparison with the two major cytoplasmic tRNA^{Tyr} isoacceptors from wheat [26] reveals that four of the nine sequenced *Triticum* tRNA^{Tyr} genes code for tRNA$_1^{Tyr}$, whereas two code for tRNA$_2^{Tyr}$ (Fig. 2). tRNA$_1^{Tyr}$ has an A:U pair at the basis of the TΨC stem and tRNA$_2^{Tyr}$ contains a G:C pair at the corresponding location. The two tRNA^{Tyr} genes encoded in pTtY7 and pTtY8 comprise a T:A instead of a C:G pair at the sixth position of the acceptor stem and are otherwise identical in their coding region with TtY4 and TtY9. The tRNA^{Tyr} gene of pTtY6 carries a C to T substitution at position 5. This nucleotide exchange leads to a U:G instead of a C:G pair in the acceptor stem of mature tRNA^{Tyr} (Fig. 2). All tRNA^{Tyr} genes bear intervening sequences of 12–14 bp length (Fig. 5A). The 5′- and 3′-flanking sequences are AT-rich as has been reported for other eukaryotic tRNA genes. The 3′-flanking regions of the *Triticum* tRNA^{Tyr} genes contain a stretch of 5–8 consecutive thymidine residues immediately following the 3′ end of the tRNA^{Tyr} genes or in its close vicinity (not shown). As a rule, at least four or more thymidines on the non-coding strand are necessary for efficient transcription termination by RNA polymerase III in eukaryotic cells [27]. No significant similarities were found in comparing the flanking sequences of all nine tRNA^{Tyr} genes with the exception of TtY1 versus TtY2 which comprise an overall homology of 65% [15].

3.3. *In vitro transcription of Triticum tRNA*^{Tyr} *genes in HeLa cell nuclear extract*

Yeast and HeLa cell nuclear extracts are the only in vitro transcription systems available so far. We have shown that most plant tRNA genes are efficiently transcribed in both extracts [13,25,28]. Here we have performed in vitro transcription of pTtY4 to pTtY9 in HeLa cell nuclear extract at 1 mM Mg^{2+} concentration. Under these conditions transcription is effective but processing and splicing of the pre-tRNAs is impaired [28]. All *Triticum* tRNA^{Tyr} genes are efficiently transcribed except for TtY6 and yielded only a single prominent product (Fig. 3). We do not know the reason for the reduced transcription efficiency displayed by TtY6. This gene is flanked by a strong terminator consisting of six thymidine residues six nucleotides downstream of the 3′ end of the gene. Possibly the 5′-flanking sequence does not support optimal initiation of transcription. In the case of TtY9, low amounts of mature tRNA are seen due to weak processing activity expressed even at a magnesium ion concentration of 1 mM (Fig. 3).

3.4. *In vitro processing and splicing of Triticum pre-tRNAs*^{Tyr} *in wheat germ extract*

We have recently established a cell-free extract from wheat germ which is active for pre-tRNA processing, splicing and modification [20]. It was later shown that in this extract introns were not excised from heterologous pre-tRNAs derived from human, *Xenopus* or yeast tRNA genes [21], but that *Nicotiana* and *Arabidopsis* pre-tRNAs^{Tyr} were good substrates for the wheat splicing endonuclease [13,28]. Hence, it was expected that maturation of homologous wheat pre-tRNAs^{Tyr} should proceed as well or even better. Apart from pre-tRNA derived from pTtY6, about 80–90% of the precursors synthesized from pTtY4 to pTtY9 were converted to mature

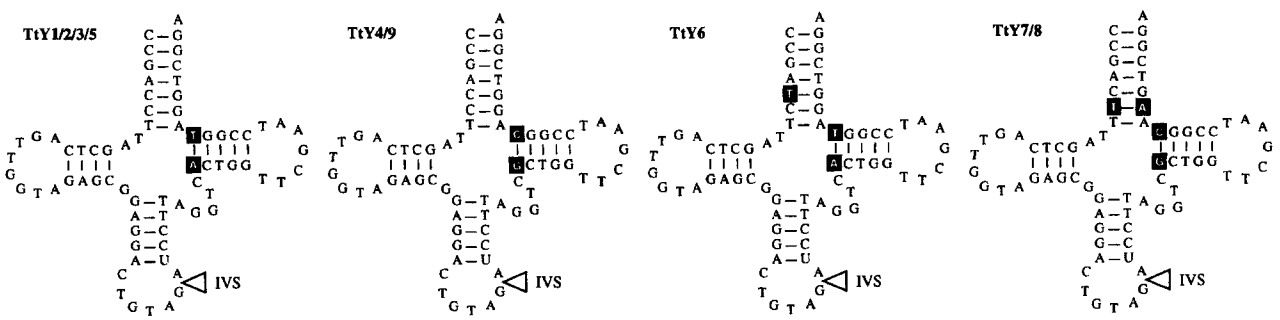

Fig. 2. Nucleotide sequences of the coding region of nine nuclear tRNA^{Tyr} genes from *Triticum aestivum* shown as cloverleaf models. The genes are grouped according to their sequence similarity. TtY1 to TtY3 have been published previously [15,22]. All genes contain intervening sequences (IVS) at the signified position. The intron sequences are presented in Fig. 5A. The nucleotides in which the four groups of tRNA^{Tyr} genes differ are indicated by white letters on a black background. The complete nucleotide sequences of the clones pTtY1 to pTtY9 are available from the EMBL database under the accession numbers X51731, X51732, X61044, X71947 to X71950, X76914 and X76915.

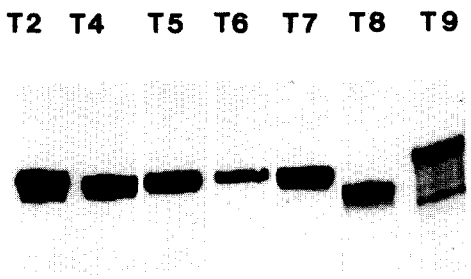

Fig. 3. In vitro transcription of *Triticum* $tRNA^{Tyr}$ genes in HeLa cell nuclear extract. The DNAs of TtY2 (T2) and TtY4 to TtY9 (T4 to T9) were transcribed in a HeLa cell nuclear extract in the presence of [α-^{32}P]GTP and 1 mM $MgCl_2$ for 60 min at 30°C. The primary products were analyzed on a 12.5% polyacrylamide/8 M urea gel. The pre-tRNAs differ slightly in size according to their variable length of leader and trailer sequences.

$tRNA^{Tyr}$ after 120 min of incubation in wheat germ extract (Fig. 4) including pre-tRNAs of TtY4 and TtY7 which are not shown here. Pre-tRNA of TtY6 is processed at a slow rate (Fig. 4B). Since we have observed that generally removal of the flanking sequences precedes intron excision in wheat germ extract [20,28] we conclude that the reduced splicing activity evident by the low amount of tRNA halves and mature tRNA is a consequence of the impaired pre-tRNA processing rather

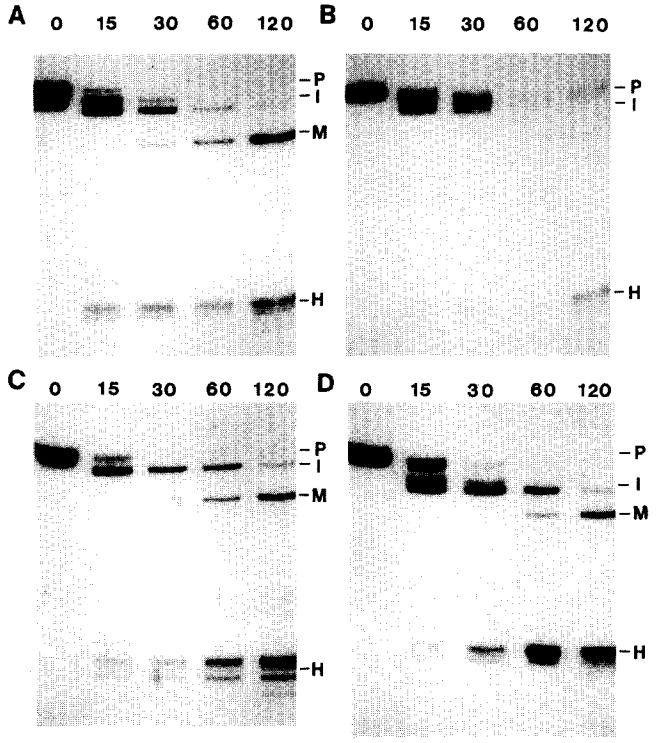

Fig. 4. In vitro processing and splicing of intron-containing *Triticum* pre-$tRNAs^{Tyr}$ in wheat germ extract. Pre-tRNAs of TtY5 (A), TtY6 (B), TtY8 (C) and TtY9 (D) were synthesized by preparative in vitro transcription of the corresponding plasmid DNAs in HeLa cell nuclear extract. The major primary transcripts, containing 5′- and 3′-flanking sequences and the intron, were recovered from the gel and were used for studying their processing and splicing in wheat germ extract. Incubation was at 30°C for the times (min) indicated above the panels. The maturation patterns of pre-tRNAs derived from pTtY1 to pTtY3 have been presented elsewhere [22,33]. P=primary transcript, I = inton-containing intermediate product, M = mature tRNA, H = tRNA halves.

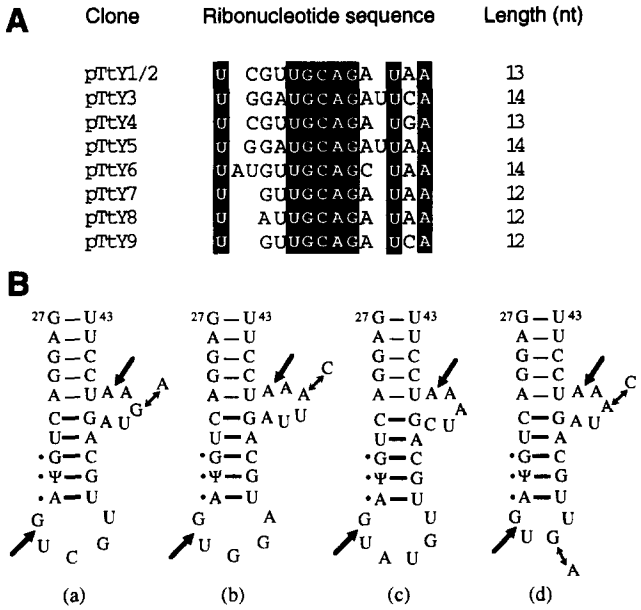

Fig. 5. Intervening sequences of nine nuclear $tRNA^{Tyr}$ genes from *Triticum aestivum*. A: The introns are aligned according to similar nucleotide sequences. Homologous nucleotides present in all introns are indicated by white letters on a black background. B: Putative secondary structures of the extended anticodon stems of *Triticum* pre-$tRNAs^{Tyr}$ TtY1, TtY2 and TtY4 (a), TtY3 and TtY5 (b), TtY6 (c), TtY7, TtY8 and TtY9 (d). The differences in nucleotide sequences of the corresponding introns are indicated. Arrows point to the 5′ and 3′ splice sites, respectively. Dots identify the anticodon.

than a deficiency in splicing activity. The mature tRNA domain of TtY6 carries a U5:G68 base pair in the acceptor stem due to a C to T transition in the corresponding gene (Fig. 2). Although G:U pairs in the acceptor stem are quite common in tRNAs and mostly do not interfere with the processing enzymes [25,29,30], single nucleotide exchanges might nevertheless impair processing reactions by leading to local helix deformations [31].

A remarkable feature of *Triticum* $tRNA^{Tyr}$ genes is the fact that they all possess introns of similar sequence and length (Fig. 5A) and as a result adopt similar secondary structures in which the two splice sites are located in single-stranded loops and are separated by five base pairs (Fig. 5B). $tRNA^{Tyr}$ genes in the *Arabidopsis* nuclear genome also contain introns of similar sequence and an identical length of 12 nucleotides. However, it should be called to mind that $tRNA^{Tyr}$ genes have been amplified at a single chromosomal site in this organism [14] whereas $tRNA^{Tyr}$ genes in the *Triticum* genome are mainly organized as a dispersed multigene family (Fig. 1). The introns of *Nicotiana* $tRNA^{Tyr}$ genes differ considerably in sequence and have a length from 13 to 25 nucleotides [13]. Obviously plant tRNA introns do not exhibit similarities on the level of primary nucleotide sequence. Yet, they have one feature in common: *Arabidopsis*, *Nicotiana* and *Triticum* pre-$tRNAs^{Tyr}$ display an extended anticodon stem formed by interactions between bases of the anticodon loop and the intron. In the case of *Arabidopsis* and *Nicotiana* pre-$tRNAs^{Tyr}$ four additional base pairs are established [13,28] whereas *Triticum* pre-$tRNAs^{Tyr}$ can even form five base pairs (Fig. 5B). These results support the general notion that plant splicing endonuclease requires a defined secondary intron structure for efficient intron excision [21,32].

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