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The mitochondrial S13 ribosomal protein gene is silent in wheat embryos and seedlings

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ABSTRACT

The sequence of a wheat mitochondrial reading frame encoding a protein homologous to the E.coli S13 small subunit ribosomal protein has been determined. The gene is located immediately downstream of a 1.4 kb recombinationally-active repeat element that contains the ATPase subunit 6 gene. The coding regions of the two genes are separated by only 153 bp, the shortest distance yet observed between protein-coding genes in plant mitochondria. However, their transcript profiles differ markedly. The ATPase 6 gene displays a single, prominent mRNA of approximately 1.4 kb, whereas the S13 gene shows no stable transcript as judged by Northern blot analysis of wheat mitochondrial RNA isolated from different developmental stages. A short segment of the 26S rRNA gene is located downstream of the S13 gene and its presence illustrates the frequent DNA duplication/rearrangements found in wheat mitochondria.

INTRODUCTION

Most eukaryotic organisms possess the same basic complement of mitochondrial genes, encoding components of the respiratory chain as well as ribosomal and transfer RNAs required for their translation. Since these proteins are essential for aerobic function, their genes are constitutively expressed in respiring cells. The only documented example of an apparently silent mitochondrial gene is the ATPase subunit 9 gene in Neurospora crassa (1) and Aspergillus nidulans (2). In vegetative cells of these fungi, the mitochondrial ATPase 9 protein is a nuclear-encoded gene product that is imported from the cytosol into the organelle. Since the mitochondrial copy of the ATPase 9 gene has all the features of a functional gene, it is believed to be expressed at some stage of the fungal life cycle.

In some organisms, ribosome-associated proteins are also encoded by the mitochondrial genome. In Saccharomyces cerevisiae and Neurospora crassa, the small subunit ribosomal proteins, var1 (ref.3) and S5 (ref.4), respectively, are mitochondrial gene products. Analogous genes are not encoded by animal mitochondrial DNA. Recently, genes showing homology to the E.coli S13 ribosomal protein gene have been identified in maize and tobacco mitochondria (5).

Northern blot analysis indicates that these genes are transcribed and have complex transcript patterns. Heterologous hybridization experiments suggest that a mitochondrial S13 gene is also present in Brassica campestris (6), located in an actively-transcribed region of the genome.

Mitochondrial transcript profiles show wide variation, both in size and complexity, among different plants. For example, those of maize mitochondrial genes are usually much more complex (cf. 5,7-9) than for the corresponding genes in Brassica campestris (6). In general, wheat mitochondrial transcripts are simple. A single, major mRNA species has been observed for each of the following wheat mitochondrial genes: cytochrome b (10), cytochrome oxidase subunit I (11), cytochrome oxidase subunit II (12), and ATPase subunit 6 (Bonen & Bird, ms. in preparation). All these genes, except that of ATPase 6, are present as single genomic copies in wheat. The ATPase 6 gene is located on a 1.4 kb repeated element that can undergo homologous recombination. Approximately ten different sets of repeated elements, each of which can give rise to various subgenomic molecules, are found on the 430 kb wheat mitochondrial genome (13). The mitochondrial genomes of other plants are also large (218-2400 kb) and usually contain a number of recombinationally-active repeat elements (cf. 14). Those mitochondrial genes that have been localized on physical maps in maize (15) and B.campestris (6), are scattered around the genome on both strands and show no conservation of gene order.

In this paper, the nucleotide sequence and transcript analysis of the wheat mitochondrial S13 ribosomal protein gene are presented. Surprisingly, this gene, which is closely linked to the ATPase 6 gene, shows no stable transcript in any of the developmental stages of wheat that have been examined.

METHODS

Wheat mitochondrial DNA (Triticum aestivum var. Thatcher) was isolated from 24 hr. germinating embryos (16) and cloned into pUC plasmid vectors (17). Mitochondrial RNA (Triticum aestivum var. Thatcher and var. Fredrick) was isolated from 24 hr. dark-grown germinating embryos, and from the coleoptile and enclosed shoot tissue of 6 day etiolated seedlings and 12 day greened seedlings, the latter having been dark-grown for the first 6 days. Isolation methods were as previously described (16) with modifications (18). Total mitochondrial RNA and the NaCl-insoluble fraction of mitochondrial RNA were used in various Northern blot experiments.

Southern and Northern blot analyses were performed using standard methods

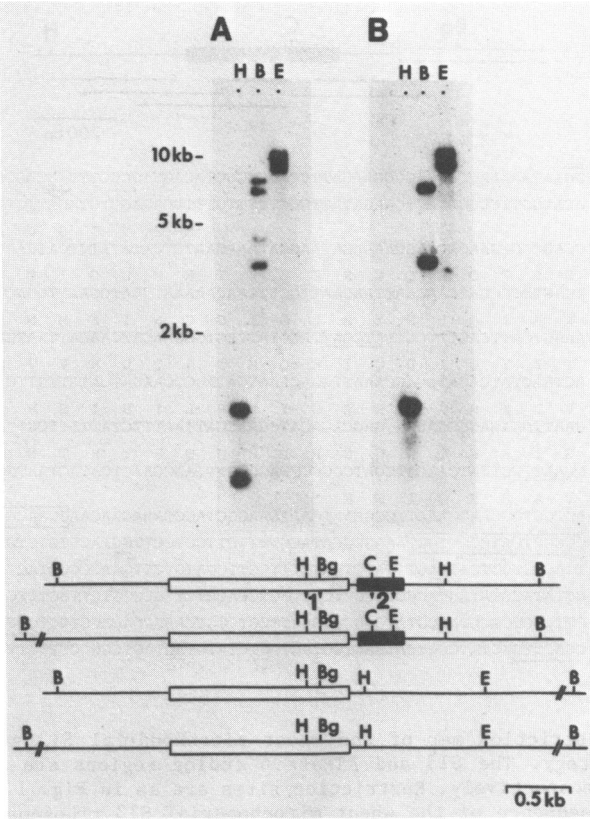

FIG. 1. Southern blot analysis of wheat mitochondrial DNA restricted with HindIII (H), BamHI (B) or EcoRI (E) and probed with (panel A) sequences internal to the ATPase 6 gene located within the 1.4 kb repeat element (open bar, probe 1); or (panel B) sequences internal to the S13 gene (black bar, probe 2). Other restriction sites: ClaI (C), BglII (Bg).

(19). RNA was fractionated on 2.2M formaldehyde, 1.25% agarose gels, transferred to Biotrans membranes (ICN) and bound to the filters by UV crosslinking. Gel conditions were chosen to retain low molecular weight RNA species. For DNA probes, M13 recombinant clones were labeled by second-strand synthesis (20) and hybridizations were conducted in 5 x SSC, 50% formamide at 42°C, overnight. Blots were washed extensively in 0.2 x SSC, 0.1% SDS at 50°C prior to autoradiography.

DNA sequencing was carried out using the dideoxy chain termination method (21), after restriction fragments had been subcloned into appropriate M13

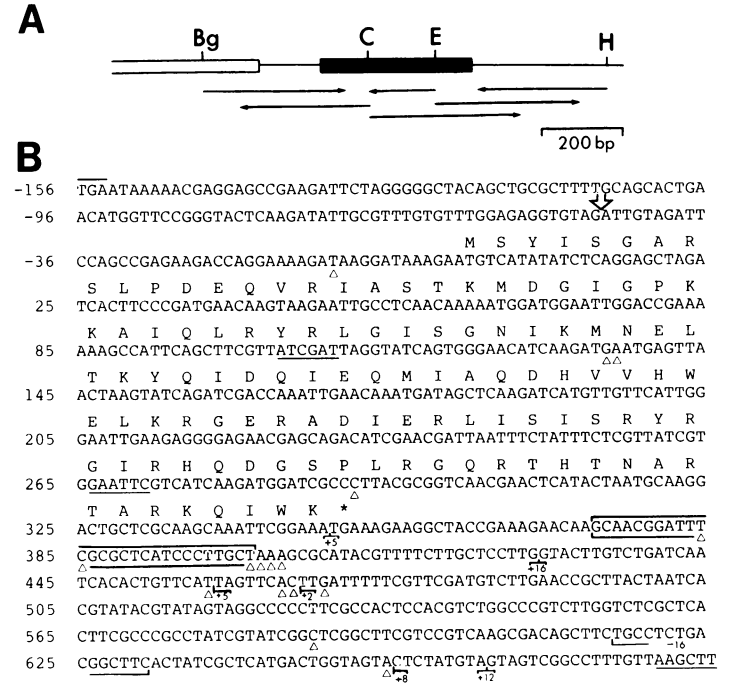

FIG. 2. (A). Restriction map of the wheat mitochondrial S13 gene region and sequencing strategy. The S13 and ATPase 6 coding regions are shown by black and open bars, respectively. Restriction sites are as in Fig. 1.
(B) Nucleotide sequence of the wheat mitochondrial S13 ribosomal protein gene and its derived amino acid sequence. Nucleotide differences between the wheat S13 gene and that of maize (5) are indicated by open triangles. Insertion/deletions are shown with their lengths. The arrow indicates the breakpoint in homology between the wheat and maize sequences, at the 3' end of the wheat 1.4 kb repeat element. The 26S rRNA gene segment is boxed. The ATPase 6 termination codon is overlined and restriction sites shown in (A) are underlined.

vectors. Data analysis was performed using the Microgenie programs (Beckman) and the GenBank and NBRF Protein Data Banks.

RESULTS

The wheat mitochondrial S13 ribosomal protein gene was identified by a computer analysis of the DNA sequence located immediately downstream of a 1.4 kb repeat element which contains the ATPase 6 gene. The schematic in Fig. 1 shows this region of the wheat mitochondrial genome and the accompanying Southern blots illustrate the pattern complexity that is seen when a structural gene is located either within (Fig. 1A) or very close to (Fig. 1B)

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WHEAT MT      MSYISGARSLPDEQVRIASTKMDGIGPKKAIQLRYRLGISGNIKMNELTKYQIDQIEQ
MAIZE MT      MSYISGARSLPDEQVRIASTKMDGIGPKKAIQLRYRLGISGNIKIHELTKYQIDQIEQ
TOBACCO MT    MLYISGARLVGDEQVRIASTKIDGIGPKKAIQVRYRLGISGNIKIKELTKYQIDQIEQ
E.COLI        MARIAGINIPDHKHAVIALTSIYGVGKTRSKAILAAAGIAEDVKISELSEGQIDTLRD
              *  * *          ** *   * *           **    *  **   ***

MIAQDHVVHWELKRGERADIERLISISRYRGIRHQDGSPLRGQRTHTNARTARKQIWK
MIAQDHVVHWELKRGERADIERLISISRYRGIRHQDGSPLRGQRTHTNARTARKQIWKGNERRLPKEQATD
MIGQDHVVHWELKRGERADIERLISISCYRGIRHQDGSPLRGQRTHTNARTCRKLIWK
EVAK-FVVEGDLRREISMSIKRLMDLGCYRGLRHRRGLPVRGQRTKTNART-RKGPRKPIKK
      **   * *     * **     *** **  * * ***** ***** **   *
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FIG. 3. Alignment of the wheat mitochondrial S13 ribosomal protein sequence with those of maize and tobacco mitochondria (5) and E.coli (23). Amino acid residues present in all four sequences are indicated by asterisks.

a recombinational repeat element. This 1.4 kb recombinational repeat element is discussed in detail elsewhere (Bonen & Bird, ms. in preparation). The data indicate two genomic copies of the 1.4 kb element, which by homologous recombination generate four genomic environments for the ATPase 6 gene, situated within the element. The S13 gene, which is a single-copy gene located immediately downstream of one copy of the 1.4 kb element, therefore, has two physical environments arising from sequence differences upstream of the repeat element.

DNA sequence analysis of the region which is downstream of the 1.4 kb element and which is located on a 3.7 kb BamHI restriction fragment, was carried out as indicated by the sequencing strategy shown in Fig. 2A. A region of approximately 1 kb was translated into all three reading frames using the standard genetic code, with the exception that CGG specify tryptophan rather than arginine (cf. 5,7-12,22). When these sequences were searched against the NBRF Protein Data Bank, homology to the E.coli S13 ribosomal protein (23) was found.

The wheat mitochondrial S13 gene and its derived protein sequence are shown in Fig. 2B. The sequence predicts a polypeptide of 116 amino acids; its initiation codon is located 46 nucleotides downstream from the end of the 1.4 kb repeat element (arrow, Fig. 2B), that is, 153 nucleotides downstream from the ATPase 6 termination codon (overlined, Fig. 2B).

The wheat sequence shows strong homology to the S13 ribosomal protein genes that have recently been characterized in maize and tobacco mitochondria (5). Only three nucleotide differences and one 5-bp insertion/deletion are seen in comparisons of the wheat and maize S13 coding regions (open triangles, Fig. 2B). Two of the nucleotide substitutions are in the third position of codons and the third is a first position change; they result in two adajacent

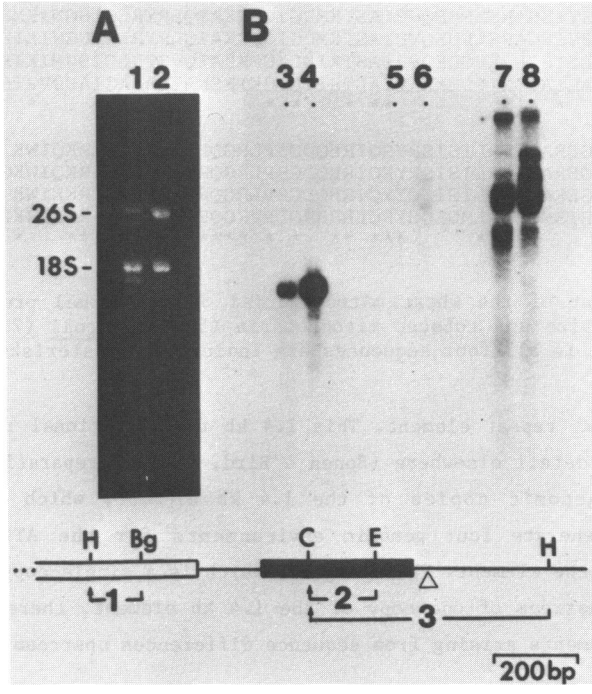

FIG. 4. RNA blot analysis of wheat mitochondrial RNA isolated from 24 hr. germinating embryos (lanes 1,3,5,7) and 6 day etiolated seedlings (lanes 2,4,6,8). (A) UV fluorescence patterns of RNA electrophoresed on 1.25% agarose/formaldehyde gels. (B) Northern blots were hybridized with probe 1 (lanes 3,4), probe 2 (lanes 5,6) or probe 3 (lanes 7,8). The schematic showing probe locations is labeled as in Fig. 1 and 2. The triangle indicates the position of the 26S rRNA gene segment.

amino acid changes (Met_{45}-Asn_{46} in wheat vs. Ile_{45}-His_{46} in maize). A 5-bp insert in the maize sequence (corresponding to nucleotide position 348 of the wheat sequence, Fig. 2B) shifts the relative position of the termination codon so that the maize mitochondrial S13 protein is predicted to be thirteen amino acids longer than that of wheat.

The wheat and tobacco mitochondrial S13 coding sequences are also closely related. They share 94% nucleotide identity and their derived protein sequences, which are of identical length, show 90% amino acid identity. An alignment of the derived S13 protein sequences from wheat, maize and tobacco mitochondria and that of E.coli (23) is shown in Fig. 3. The wheat mitochondrial and E.coli S13 proteins share 38% amino acid identity and all four proteins display particularly strong similarity in the carboxy terminal

region. They all possess a high proportion of hydrophilic residues consistent with the known globular structure of the E.coli S13 protein (24).

The regions immediately flanking the mitochondrial S13 gene are highly conserved between wheat and maize. In the 5' flanking region, only one nucleotide difference, out of 46 positions, is seen (open triangle, Fig. 2B). Farther upstream, the wheat and maize sequences are unrelated; the breakpoint in homology coincides with the 3' end of the wheat 1.4 kb repeat element. As observed for other wheat mitochondrial protein-coding genes (10-12), the initiation codon of the S13 gene is preceded by a purine-rich stretch. In the 3' flanking region shown in Fig. 2B, the wheat and maize sequences share 95% nucleotide identity. Most of the differences are short insertion/deletions rather than nucleotide substitutions and such inserts are often flanked by short direct repeats, suggestive of slipped strand mispairing during replication. When the wheat and tobacco mitochondrial S13 flanking sequences are compared, no significant homology is seen in the 5' region, whereas two stretches of near identity, interrupted by a 54 bp insert in the wheat sequence, are found in the 100 bp region following the wheat S13 gene.

One interesting feature of the sequence downstream of the wheat mitochondrial S13 gene is the presence of a stretch (boxed, Fig. 2B) that is almost identical (27/28 nucleotides) to an inverted segment of the 26S ribosomal RNA gene (Spencer, Schnare and Gray, personal communication). This block corresponds to the complement of positions 2315 to 2342 in the published sequence of the maize mitochondrial 26S rRNA gene region (25). A closely-related sequence is present at the analogous position in maize (Fig. 2B), but not in tobacco. The latter has a 54 bp deletion relative to wheat and maize in this region.

To examine the transcriptional activity of the wheat mitochondrial S13 gene, mitochondrial RNA isolated from 24 hr. germinating embryos, 6 day etiolated seedlings and 12 day greened seedlings was hybridized to an S13 gene-internal probe in Northern blot experiments. In no case was a stable transcript seen (Fig. 4, lanes 5 & 6; and data not shown). The Southern hybridizations shown in Figure 1 served as controls because the same probes were used in these experiments. In contrast to the absence of S13 transcripts, a prominent ATPase 6 mRNA of approximately 1.4 kb was observed in all cases (Fig. 4, lanes 3 & 4). Its steady state level appears to be considerably higher in the 6 day seedlings than in the 24 hr embryos. However, levels were not quantitated because the degree of contaminating cytosol and chloroplast RNA in the mitochondrial RNA preparations was not determined.

When a probe that contains 3' flanking sequences in addition to the S13 coding region was used, a complex pattern of stable transcripts was seen (Fig. 4, lanes 7 & 8). At least part of the hybridization to an RNA species of 3.5 kb can be attributed to the presence of the short 26S rRNA segment in the probe. This was ascertained by using a probe that maps farther downstream (data not shown). The intensities of signals arising from RNA species of 3.5 kb in length are approximately equal in blots of wheat embryo and seedling mitochondrial RNA, whereas the relative levels of several higher molecular weight transcripts are higher in the seedlings than in the embryos (Fig. 4, lanes 7 & 8).

DISCUSSION

The wheat mitochondrial S13 ribosomal protein gene is separated from the ATPase 6 gene by only 153 nucleotides, yet the transcript profiles of the two genes are markedly different. The ATPase 6 gene shows a single, prominent mRNA of approximately 1.4 kb whereas the S13 gene shows no stable transcript in any of the developmental stages examined (24 hr. germinating embryos, 6 day etiolated seedlings or 12 day greened seedlings). This implies that transcription either terminates within the intergenic region or that transcripts containing the S13 coding region are preferentially degraded. Very little is known, as yet, about the mechanisms that control transcription, RNA processing, or mRNA stability in plant mitochondria. However, it appears that secondary structural motifs such as those observed at the 3'termini of some maize and Oenothera mitochondrial transcripts, (26) are not present in the region between the wheat ATPase 6 and S13 genes.

Although the wheat mitochondrial S13 gene is apparently not constitutively expressed, comparative DNA sequence analysis provides a strong argument that the gene is in fact functional. Its coding region is almost identical (99% nucleotide identity) to the actively-transcribed S13 gene found in maize mitochondria (5). This level of homology is comparable to that observed between other wheat and maize mitochondrial protein-coding genes (10-12). Moreover, as was also noted by Bland et al. in their comparison of the maize and tobacco mitochondrial S13 genes (5), the abundance of third position codon changes compared to those in first or second positions, and the conservative nature of most amino acid substitutions suggest a strong evolutionary constraint on the gene. In this regard, the wheat mitochondrial S13 derived protein and that of E.coli share 38% amino acid identity, a value considerably higher than that observed between the comparable ATPase 6 protein sequences

(cf. 18% amino acid identity, Bonen & Bird, ms. in preparation). In E.coli, the S13 protein is a globular, basic protein that interacts with RNA, other ribosomal proteins and perhaps initiation factors, near the interface between the two ribosomal subunits (reviewed in ref. 24). The wheat, maize and tobacco S13 sequence data are consistent with the presence of an analogous S13 protein in plant mitochondrial ribosomes. Such similarities between plant mitochondrial and E.coli ribosomal proteins are not unexpected since their ribosomal RNAs are known to share a number of distinctive primary and secondary structural features (27). Such data constitute persuasive evidence for the endosymbiotic origin of plant mitochondria.

The two most likely explanations for the absence of stable S13 transcripts in wheat mitochondria are (a) that the gene is only expressed within a certain period of the wheat life cycle or (b) that it very recently lost its ability to be expressed but the DNA sequence has not yet accumulated mutations which would convert it to a pseudogene. The first hypothesis has previously been proposed to rationalize the presence of a silent ATPase 9 gene in Neurospora and Aspergillus mitochondria (1,2). If this is the case for the S13 gene under discussion here, then the S13 protein located in the mitochondrial ribosome, assuming such a protein is required for translation, must be encoded by another gene. Since no S13-homologous sequences have been identified in the tobacco or liverwort chloroplast genomes, both of which have been completely sequenced (28,29), the most likely source is a nuclear gene. This would be analogous to the situation for the fungal mitochondrial ATPase 9 protein. In Neurospora, the nuclear-encoded ATPase 9 protein found in the mitochondria, shares 56% amino acid identity with the derived protein sequence of the silent mitochondrial copy (1). It is interesting that the analysis of pea, bean and soybean mitochondrial DNA in heterologous hybridization experiments (ref.5 and data not shown), has not revealed the presence of S13-homologous sequences. The S13 ribosomal protein may therefore be derived solely from a nuclear gene in those plants. This would imply that various gene transfer events, resulting in the acquisition or loss of S13 genes, have occurred in different plant lineages. The very close sequence relationship among the wheat, maize and tobacco mitochondrial S13 genes strongly suggests that the gene was present in their common ancestor.

A comparison of the wheat and maize S13 flanking sequences does however indicate that rearrangement events have occurred during the estimated 50-70 million years (30) since the time of divergence of those two plants. The 5' flanking sequences are almost identical for only 46 nucleotides; homology

abruptly ends at the position of the wheat 1.4 kb repeat element. It is possible that DNA duplication/rearrangements of the 1.4 kb element have altered the genomic environment of the S13 gene so that it no longer possesses expression signals needed for transcription initiation or RNA processing. If so, such events presumably have involved more than the simple insertion of the 1.4 kb element into the S13 promoter region since no sequence similarity is observed between the maize S13 upstream region and sequences immediately preceding either copy of the wheat 1.4 kb element (data not shown). Moreover, such DNA rearrangements must have occurred very recently, since the wheat S13 coding region has apparently not accumulated any deleterious nucleotide substitutions. It is anticipated that the mitochondrial DNA arrangement observed in a given wheat cultivar is stable throughout its life cycle, since previous studies have revealed no developmentally-specific or organ-specific differences in wheat mitochondrial DNA restriction profiles even among different cultivars (31). Regardless of the reason for the lack of expression of this gene, these observations suggest the presence of another S13 gene in wheat, either within the mitochondrion or the nucleus, to provide a functional ribosomal protein.

The close physical linkage of the wheat mitochondrial S13 and ATPase 6 genes is unlike the gene organization observed for these, or any other, genes that have been mapped in maize (15) or Brassica (6). In those plants, the genes are far apart and both the ATPase 6 and S13 genes are present as single genomic copies. The most closely-linked plant mitochondrial protein-coding genes previously reported are the tobacco ATPase subunit 9 and S13 genes, which are 478 bp apart (5). Northern analysis indicates that these genes are co-transcribed, with several stable RNA species containing the coding regions of both genes. In yeast and animal mitochondria, when closely-linked genes are co-transcribed, they are usually processed into separate mRNAs (32,33). If the wheat mitochondrial S13 and ATPase 6 genes are co-transcribed, then the S13 region of the transcript must be unstable.

Sequence analysis of the wheat mitochondrial S13 gene also revealed the presence of a stretch of 28 nucleotides that is identical, with one mismatch, to a segment of the 26S rRNA gene. It is located 22 nucleotides downstream of the wheat S13 termination codon. In maize, because the mitochondrial S13 gene is 39 bp longer than that of wheat, this block constitutes part of the S13 coding sequence and specifies the termination codon. Such pieces of mitochondrial genes have been observed previously in wheat mitochondria, notably a segment corresponding to the transmembrane domain of the wheat

cytochrome oxidase subunit II protein (12) and an internal region of the cytochrome b gene (10). The presence of gene segments contributes to the complexity of plant mitochondrial DNA and if located near other genes, such sequences can contribute to the complexity of transcript patterns. Gene segments can also give rise to novel, chimeric plant mitochondrial genes and such products of DNA duplication/rearrangements have been implicated in the phenomenom of cytoplasmic male sterility (34,35).

The transcript analysis of the wheat mitochondrial S13 gene region supports the idea that certain wheat mitochondrial genes are differentially expressed. Discrete, high molecular weight transcripts arising from the region downstream of the S13 gene and extending close to, but not into, the S13 coding region, show different patterns in wheat embryos and seedlings. An internal segment of the wheat mitochondrial NADH dehydrogenase subunit I gene is located approximately 1 kb downstream of the S13 gene (data not shown), as in maize and tobacco (5), and a detailed transcript analysis of this region is currently under investigation. Although developmentally-specific gene expression is rarely seen in fungal or animal mitochondrial systems, the biosynthesis of developmental stage-specific polypeptides has been observed in maize mitochondria (36).

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