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Changes in the content of modified nucleotides in wheat rRNA during greening

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Abstract

The modified nucleotide content of the ribosomal RNAs in wheat is greatly influenced by light. The rRNAs of etiolated seedlings contain far fewer modified derivatives. The modified nucleotide composition characteristic of green plants develops gradually as a result of irradiation. In the course of the experiments changes in the state of modification of 5.8S and 18S rRNAs were examined during the greening of etiolated wheat seedlings. Three types of minor nucleotides, $O^{2'}$-methyladenosine, $O^{2'}$-methylguanosine and pseudouridine were found in the 5.8S rRNA of green wheat leaves, none of which was detected in etiolated wheat. The minor nucleotides appeared in the 5.8S rRNA only after 48 h irradiation. The sequences of 5.8S rDNA, ITS1, ITS2 and 18S rDNA were also determined and the presence of the hyper-modified nucleotide 1-methyl-3-(α-amino-α-carboxypropyl)-pseudouridine was detected in green wheat 18S rRNA. This minor component was not demonstrable in etiolated wheat 18S rRNA, but appeared after irradiation for 48 h.

Additional key words: 5,8S rRNA, 18S rRNA, ITS1 and ITS2, hyper-modified pseudouridine.

Introduction

In addition to the four basic nucleotides, ribosomal RNAs contain a large number of modified (minor) nucleotides. These components play an important role in the effective functioning of the RNAs, providing the fine controls of their physiological role. Eukaryotic ribosomal RNAs contain considerably larger numbers of modified nucleotides than their prokaryotic counterparts (Brand *et al.* 1978, Cecchini and Miassod 1979, Maden 1990, Venema and Tollervey 1999). Within the eukaryotes, higher plants again have a higher ratio of minor nucleotides than lower plants. The ratio and composition of the minor nucleotides change as a function of physiological processes (Rácz *et al.* 1978, 1983, Lásztity *et al.* 1991).

The minor nucleotides found in eukaryotic ribosomal RNAs can be divided into three groups. The first contains pseudouridine (Ψ) derivatives, which make up some 40 % of the rRNA minor nucleotides. The second group consists of O-methylated derivatives, comprising a further 45 - 50 % of the total modified nucleotides. Low molecular mass nucleolar RNAs and modifying enzymes play an important role in the biosynthesis of these two groups of minor nucleotides (Brand *et al.* 1978, Bousquet-Antonelli *et al.* 1997, Bachellerie and Cavaille 1997). The third group is made up of basically methylated and hyper-modified derivatives with complex structure.

The structure of wheat 5.8S rRNA was determined by Mackay *et al.* (1980). Parallel to the determination of the primary structure, 4 minor nucleotides were also demonstrated: pseudouridine at positions 22 and 78, O-methylated adenine (Am) at position 47 and O-methylated guanine (Gm) at position 79. Previous studies demonstrated that far fewer modified nucleotides could be detected in the ribosomal RNAs and tRNAs of etiolated wheat leaves than in the relevant RNAs of green wheat. The state of modification characteristic of the green plant develops gradually as a result of irradiation

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Abbreviations: Am - $O^{2'}$-methyladenosine, Gm - $O^{2'}$-methylguanosine, ITS1, ITS2 - intragenic spacer regions, $m^1acp^3\Psi$ - 1-methyl-3-(α-amino-α-carboxypropyl)-pseudouridine.
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(Rácz *et al.* 1978, 1983, Lásztity *et al.* 1991). Studies were thus made on which minor nucleotides are present in the 5.8S rRNA of etiolated plants and how this minor nucleotide composition changes in the course of greening.

Materials and methods

Plants: *Triticum aestivum* L. (cv. MV 15) seedlings were grown in 1 mM $CaSO_4$ for 3 d and then in Knop medium. Control (light-grown) plants were cultivated from the third day on, under a 17-h photoperiod, under fluorescent and incandescent lamps giving an irradiance of 300 μmol m^{-2} s^{-1}, while other plants were irradiated for 24 and 48 h immediately before harvest.

Isolation and purification of RNA: The isolation of RNA was carried out using the phenol method as described by Kirby (1968). Total RNA was extracted by 2 M NaCl. The soluble fraction contained low molecular mass 5S and 5.8S rRNA and tRNA. The 5.8S rRNA was separated on a benzoylated diethyl-aminoethyl-cellulose column (BD-cellulose chromatography) containing 10 mM $MgCl_2$, using a linear NaCl gradient (0.425 - 0.6 M) (Gillam *et al.* 1967). The RNA was further purified on a *Sephadex G-200* column. The purity of the sample was tested by means of 15 % polyacrylamide gel electrophoresis according to Loening (1967).

The 18S rRNA was separated from the total rRNA on a 420 × 35 mm *Sepharose 4B* chromatographic column as described by Cornelis (1978). The purity of the fractions was checked by means of polyacrylamide gel electrophoresis (Loening 1967).

Hydrolysis of RNA and identification of minor nucleotides: The acid and enzyme hydrolysis of 18S rRNA and *Dowex 1×8* anion exchange chromatography of hydrolysates were carried out as described earlier (Rácz *et al.* 1978, 1983). Thin-layer chromatography of the fractions of *Dowex 1×8* anion exchange chromatography was carried out according to Björk and Swensson (1967). Minor nucleotides were identified on the basis of their UV spectra. 1-methyl-3-(α-amino-α-carboxypropyl)-pseudo-uridine was identified by means of thin-layer chromatography as described by Cecchini and Miassod (1979).

Amplification and sequencing of DNA: Isolation of DNA was carried out using the method of Murray and Thompson (1980). PCR reactions were carried out in 0.05 cm^3 of buffer containing 2 μg of the template DNA, 1.25 U of *Taq* DNA polymerase (*Perkin Elmer,* Foster City, USA), 10 mM Tris-HCl, pH 9 (25 °C), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM dNTPs and 10 pmol of the primers. The following reaction temperature cycles were used for the amplification of the 18S rDNA: 98 °C for 3 min; then 94 °C for 0.5 min, 44 °C for 0.45 min and 72 °C for 2 min for thirty cycles; followed by a final extension at 72 °C for 7 min. The sequences of the oligonucleotide primers used for the PCR amplifications were: 5'-AACCTGGTTGATCCTGCCAG (1 - 20 forward positions) and 5'-GCTTGATCCTTCTGCAGGTTCACCTAC (1791 - 1765 reverse positions). For the amplification of the total ITS region the following reaction temperature cycles were used: 94 °C for 5 min; then 94 °C for 0.5 min, 53 °C for 0.5 min and 72 °C for 0.5 min (1 s added in each cycle) for thirty cycles; followed by a final extension at 72 °C for 4 min. The sequences of the primers were: 5'-TCCGTAGGTGAACCTGCGG and 5'-TCCTCCGCTTATTGATATGC (White *et al.* 1990).

Amplification products were purified with the Prep-A-Gene kit (*Bio Rad,* Hercules, USA) and used for sequence analysis. The DNA sequence was obtained by the dideoxynucleotide chain termination method using BigDye™ Terminator Cycle Sequencing Kit (*PE Applied Biosystems,* Foster City, USA) and an automatic sequencer (*ABI PRISM 310 Genetic Analyser, Applied Biosystems*) under conditions recommended by the manufacturer. Oligonucleotides used for the sequencing reactions of the 18S rDNA were those used for the amplification and also: 5'-(C)/(T)GA(C)/(T)T(C)/(T)CGGAGAGGGAGCCTG, 5'-(C)/(T)AGAGGTGAAATTCTTGGAT and 5'-GGTGGTGGTGCATGGCCGTTC (Saunders and Druehl 1992). Primers used for the direct sequencing of the ITS1, 5.8S rDNA and ITS2 were those used for the amplification.

DNA sequences of 18S rRNA gene and total ITS region (ITS1, 5.8S rDNA and ITS2) of *Triticum aestivum* were deposited into EMBL with the accession numbers AJ272181 and AJ301799, respectively. Multiple alignments were constructed with the program *ClustalW* at the EMBL web site.

Results

After separating the 5.8S rRNA hydrolysate of green plants using two-dimensional thin layer chromatography, four modified nucleotides were identified: two pseudouridine, Gm and Am, in agreement with data

TCGTGACCCTGACCAAAACAGACCGCGCACGCGTCATCCAATCCGTCGGTGACGGCACCGTCCGTCGCT

CGGCCTATGCCTCGACCACCTCCCCTCCATCGGAGAGGGTGGGGGCTCGAGGCAAAAGAACCCACGGCG

CCGAAGGCGTCAAGGAACACTGTGCCTAACACGGGGCGCATGGCTAGCTTGCTAGCTGTGACTCGTGTT

GCAAAGCTATTTAATCCACACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAG

CGAAATGCGATACCTGGTGTGAATTGCAGAATCCCGCGAACCATCGAGTCTTTGAACGCAAGTTGCGCC

CGAGGCCACTCGGCCGAGGGCACGCCTGCCTGGGCTGTCACGCCAAAACAGGTTCCNACANCCCTCATT

GGGAATCGGGATGCGGCATCTGGTCCCTCGTCTCGCAAGGGACGGTGGACCGAAGTTAGGGCTGCCGGC

GTACCGTGTCGAACACAGCGCATGGTGGGCGTCTTTGCTTTATCAACTGCAGTGCATACGACGCGTAGC

CGGCATTATGGCCTCANAACGACCCAACAAACGTAGCGCACGTCGCTTCGACC

Fig. 1. Sequence of wheat seedling rDNA ITS1, 5.8S rDNA (*shaded*) and ITS2 regions.

presented by Mackay *et al.* (1980). No minor bases could be isolated from the hydrolysate of the 5.8S rRNA from etiolated plants or from plants illuminated for 24 h. After 48 h irradiation the minor base composition of the 5.8S rRNA was identical to that of green plants, *i.e.* two Ψ, Am and Gm were isolated. Determinations were also made on the sequences of 5.8S rDNA and the relevant ITS1 and ITS2 regions. (Fig. 1).

With the aid of *Sepharose 4B* ion exchange column chromatography it proved possible to isolate wheat 18S rRNA as a separate fraction, so a new attempt was made to identify the derivative. Simultaneously the primary structure of the 18S rRNA gene was determined.

Table 1. Rf values for thin layer chromatography with solvents isopropanol/cc. HCl/water 68:17:14.4 (v/v). U - uridine, Up - uridine monophosphate, Ψ - pseudouridine, Ψp - pseudouridine monophosphate, $m^1acp^3\Psi$ - 1-methyl-3-(α-amino-α-carboxypropyl)-pseudouridine.

Compound	Rf value
Up	0.72
Ψp	0.58
U	0.59
Ψ	0.43
$m^1acp^3\Psi$	0.40

When the acid and enzyme hydrolysis products were separated by means of *Dowex 1×8* anion exchange column chromatography the fourth fraction eluted contained the pseudouridine derivatives in case of plants irradiated for 48 h (Fig. 2*C*). This was in good agreement with our earlier results (Rácz *et al.* 1978, 1983).

The components of the peak were separated with thin layer chromatography. We applied the 68:17:14.4 isopropanol/HCl/water mixture used by Cecchini and Miassod (1979) to separate the hyper-modified uridine derivatives in studies on the modified nucleotide composition of *Acer pseudoplatanus* 17S rRNA. Pseudouridine and O-methyl-pseudouridine were identified on the basis of their Rf values and absorption spectra and $m^1acp^3\Psi$ was identified on the basis of its Rf value (Table 1). It was found that the Rf value of this minor component was 0.4, which corresponded to that of $m^1acp^3\Psi$. This hyper-modified derivative could not be detected in corresponding peaks *Dowex 1×8* profile of 18S rRNA hydrolysate isolated from etiolated, or 24 h illuminated plants (Fig. 2*A,B*). It only appeared after 48 h irradiation.

The sequence of 18S rDNA (1764 bp) was also determined and the probable location site of $m^1acp^3\Psi$ was suggested (Fig. 3.) on the basis of results described by Maden (1990).

Discussion

The 5.8S rDNA sequence data agreed with those reported by Mackay *et al.* (1980), but differences amounting to around 7 % were observed in the ITS1 and ITS2 sequences. The ITS regions of rDNA are more variable than the gene of 5.8S rRNA

The modified nucleotide content of 5.8S rRNA from control green wheat seedlings was also found to be similar to the modification pattern described by Mackay *et al.* (1980), *i.e.*, two Ψ, Am and Gm were identified. The appearance of modification proved to be light dependent. Etiolated plants did not contain modified nucleotides and they appeared only after 48 h of illumination.

The ribosomal 18S RNAs contain complex derivatives with complicated structures, the best known of which is 1-methyl-3-(α-amino-α-carboxypropyl)-pseudouridine ($m^1acp^3\Psi$). This compound was first isolated by Saponara and Enger (1974) from the

18S rRNA of Chinese hamsters, and was later detected in numerous other organisms (*e.g.*, yeast, mammals, and plants) (Cecchini and Miassod 1979, Brand *et al.* 1978, Maden 1990).

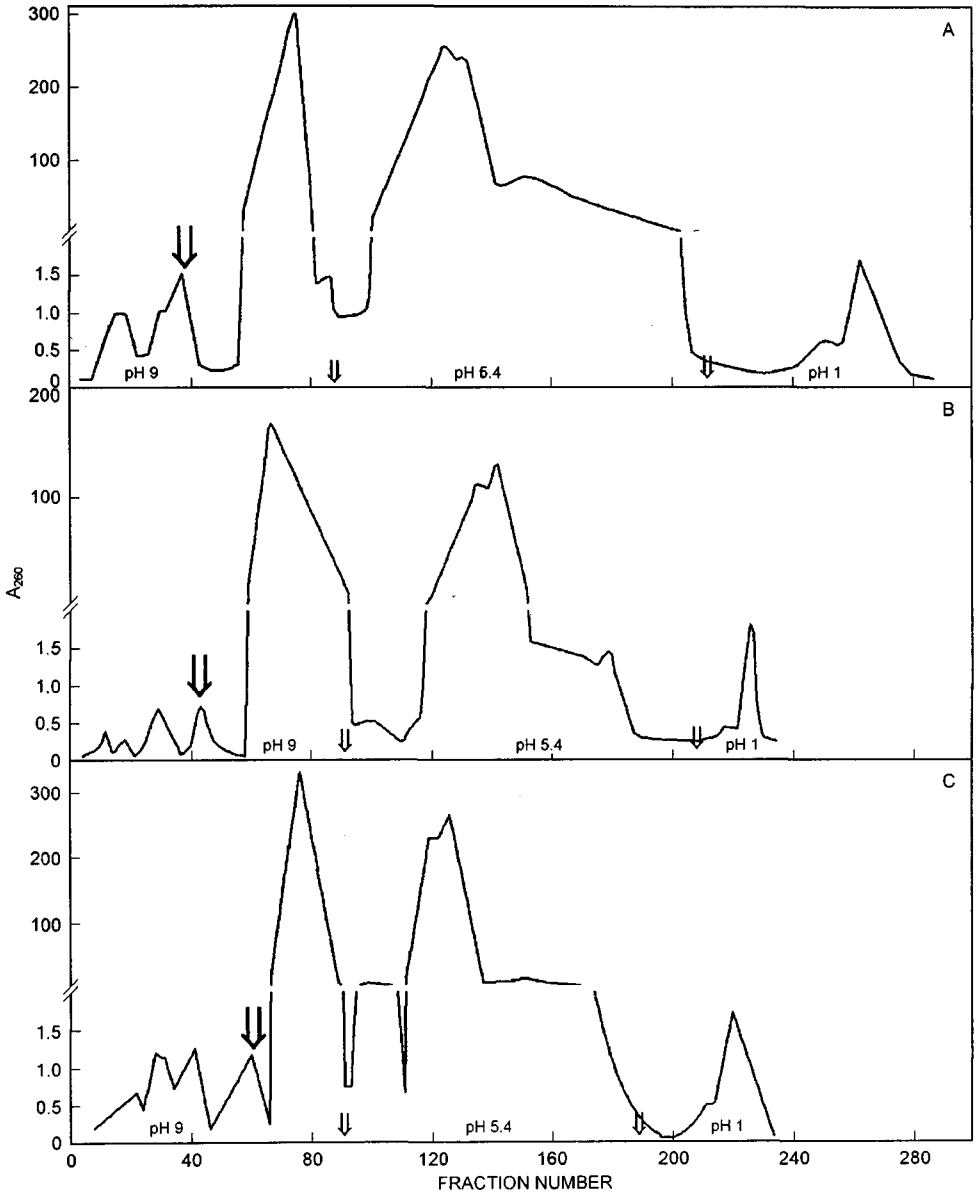

Fig. 2. *Dowex 1×8* elution profile of an acidic hydrolysate of 18S rRNA of etiolated (*A*), 24 h irradiated (*B*) and 48 h irradiated (*C*) wheat seedlings. *Arrows* indicate TLC analysed peaks.

In our previous study on the minor base composition of wheat 18+26S rRNA the presence of this modified nucleotide was not detected (Rácz *et al.* 1983) probably because it is present in the 18+26S rRNA fraction in very small quantities. In our present work we isolated wheat 18S rRNA as a separate fraction, and identified $m^1acp^3\Psi$ in hydrolysis products on the basis of its Rf value. According to our results it appears that hyper-modified Ψ is present also in wheat 18S rRNA. This derivative could not be detected in 18S rRNA of etiolated or 24-h-illuminated seedlings. It only appeared after 48 h illumination, together with methyl-pseudouridine. These data are in good agreement with those recorded for 18+26S rRNA, where no pseudouridine was detected in etiolated plants, but Ψ appeared after 48 h illumination (Rácz *et al.* 1983). This is supported by the fact that the synthesis of pseudouridines and *O*-methylated derivatives takes place in eukaryotic ribosomal RNAs with the aid of small nucleolar RNAs (snoRNA) and modifying enzymes (Bousquet-Antonelli *et al.* 1997, Bachellerie and Cavaillé 1997). The expression of genes of snoRNAs and/or modifying enzymes could also be regulated by light.

The results obtained during greening were in close correlation with data recorded in studies on changes in the degree of modification and functional activity of wheat rRNAs (Rácz *et al.* 1983, Lásztity *et al.* 1991).

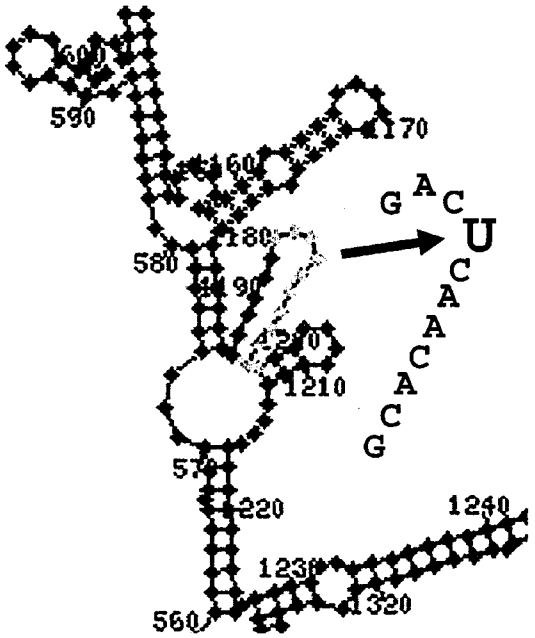

Fig. 3. Part of the wheat 18S rRNA secondary structure with the lowest initial minumum free energy (dG) - 2854.3 kJ mol^{-1} computed by mfold version 3.1 (folding temperature: 37 °C, ionic conditions: 1 M NaCl, no divalent ions) according to Zuker *et al.* (1999) and Mathews *et al.* (1999). Bases with different tone represent the T1 RNAse fragment identified by Maden (1990) where the hypermodified 1-methyl-3-(α-amino-α-carboxypropyl)-pseudo-uridine ($m^1acp^3\Psi$) is localised in the 1196th position.

The sequence of 18S rDNA of wheat was also determined. Sequence homologies were used to locate the probable site of the hyper-modified derivative. This is probably the position 1196 (Maden 1990). When the sequence was compared to that of related species it showed the greatest similarity to *Hordeum jubatum*.

It can be concluded from the results of our present and previous studies that the development of post-transcriptional modification in the rRNAs of higher plants is a light-dependent process, taking place gradually as the seedlings turn green. In our previous papers, we found that 18S + 26S rRNAs contain low contents of minor nucleotides in etiolated wheat seedlings. RNAs of plants illuminated for 24 h contain more minor nucleotides than those of etiolated seedlings, but less than those of light grown ones. The appearance of the characteristic minor nucleotides needs a rather long period of at least 48 h (Rácz *et al.* 1978, 1983). The same was observed in the present study in the case of 5.8S RNA, which indicates the operation of similarly regulated modifying machinery at these rRNAs. The presence or absence of modified nucleotides in various types of rRNAs influences their biological activity and their efficiency in the protein synthesising system. Previously we detected decreased level of protein synthesis in the presence of undermodified rRNAs in *in vitro* protein synthesising system (Lásztity *et al.* 1991, 1999). It is known, that the 5.8S RNA takes part in the function of peptidyl transferase center in the ribosomes (Venema *et al.* 1999). The absence of modified nucleotides in this RNA can also be supposed to contribute to the decreased activity of protein synthesising system, since the minor bases provide a form of fine tuning of protein synthesis in living organisms.

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