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METHYLATION PATTERN OF RIBOSOMAL RNA GENES IN NOR-DELETED AND NOR- RECONSTRUCTED BARLEY LINES (*HORDEUM VULGARE* L.). ORGANIZATION OF IGS IN rDNA REPEAT UNIT

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Summary. The methylation pattern of rRNA genes in reconstructed barley karyotypes (*Hordeum vulgare* L.) with altered position or structure of the two nucleolus organizers (NORs) was studied by double digestion with EcoRI and HhaI and molecular hybridization with specific rDNA probes. The lack of the whole rRNA gene cluster residing in chromosome 6H in the homozygous deletion line T-35 led to an increased hypomethylation of the rRNA genes in the remaining NOR5H. On the other hand, repositioning by translocation of the distant part of the split NOR6H to the short arm of the chromosome 5H in the translocation line T-21 did not correlate with any significant changes in the methylation of –GCGC– sequences in rDNA units. In addition, the length of the intergenic spacer (IGS) in both reconstructed barley lines T35 and T21 was also analyzed. Our results showed the same length of IGS in the long rDNA repeat as compared with previously published rDNA clone (GenBank HQ825319). However, the short IGS of studied barley lines was about 500 bp longer than that published previously (GenBank AF1475501).

Key words: rRNA genes; intergenic spacer (IGS); rDNA methylation; barley

INTRODUCTION

In eukaryotes, ribosomal RNA (rRNA) genes are organized in large tandemly repeated blocks at one or a few chromosome locations (Long and Dawid, 1980). Each repeat includes a single rDNA transcription unit that codes for the 18S, 5.8S and 25–28S rRNA genes as well as the internal transcribed spacer 1 and 2 (ITS). An intergenic spacer (IGS) region separates the transcription units of the adjacent repeats. The rRNA genes, which are transcribed by the RNA polymerase I, are highly conserved throughout plants, animals, and fungi (Gruendler et al., 1991; Zentgraf and Hemleben, 1992). In contrast, the IGS, which consists of the sequences between the 3′-end of 25S rRNA and 5′-end of 18S rRNA, is more variable in both

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length and structure organization even between closely related species (Allard et al., 1990; Pikaard, 2000, 2002; Preuss and Pikaard, 2007). However, the functional role of IGS is conserved, containing a transcription initiation site (TIS), a transcription termination site (TTS), and different *cis*-regulatory elements (Grabiele, 2011). IGS itself is flanked by external transcribed spacers (ETS) (Beven et al., 1996). The 5′ ETS sequence is very long in mammals (Renalier et al., 1989), whereas it is much shorter in plants and yeast (Shaw et al., 1995).

The intergenic rDNA spacers could be substantially variable in size due to differences in the number of repetitive elements among the closely related species (Pikaard, 2000, 2002; Preuss and Pikaard, 2007; Carvalho et al. 2011). Intraspecific IGS length variability is generally associated with different numbers of motifs referred to as subrepeats. The IGS variants are not randomly distributed and usually correspond to specific chromosome locations (Appels and Dvorak, 1982; Allard et al., 1990; Carvalho et al., 2011). The variation in the number of subrepeats alters the length of the entire IGS region leading to the occurrence of spacer length variants that can be detected by restriction enzyme digestion coupled whit Southern hybridization (Allard et al., 1990).

DNA methylation is considered to be a key mechanism in the regulation of gene expression operating at both transcriptional and posttranscriptional levels. Ribosomal RNA genes in plants seem to be heavily methylated. It has been shown that the degree of methylation of rRNA genes in wheat correlates with the activity of the NORs (Flavell et al., 1988; Carvalho et al., 2010). The distribution of unmethylated cytosine residues along the repeated rDNA units is not random, but depends on the activity of the corresponding NORs (Flavell et. al., 1988). Furthermore, some specific sites in the repeated rDNA unit were found to be regularly hypomethylated (Flavell et al., 1988). Special interest deserves IGS of rRNA genes where the promotor region and transcription termination site (TTS) as well as other repetitive elements are localized and they are supposed to play an important role in the regulation of transcription (Tucker et al., 2010).

In barley there are two co-dominant classes of ribosomal repeats, which differ in length of their IGS localized in two separate loci on chromosomes 6H and 5H (Gerlach et al., 1979). A single restriction enzyme EcoRI site located in the 3′-end of the 25S rRNA gene determines the varying length of rRNA genes in NOR5H and NOR6H (8.8 and 9.8 kb, respectively). It is well known that when both NORs were combined by reciprocal translocation on the same chromosome, the activity of the transposed NOR was reduced (Nikoloff et al., 1977). This NOR suppression was not complete since the secondary constriction remained intact and the underdominant locus formed a micronucleus while the dominant locus formed a normal one. Karyotype T-35 is one of the very few examples in diploid plants, where the loss of a large cytologically visible chromosome segment in homozygous condition does not affect the viability and fertility of the plants. Cytological and molecular analyses revealed that T-35 contains a deletion which includes the whole rRNA gene cluster residing in the NOR of chromosome 6H. The deletion

of this gene family was found to be accompanied by a marked increase in the activity of the remaining NOR located on chromosome 5H (Gecheff et al., 1994). It was clearly shown that the compensatory effect in T-35 was due to an increased rate of transcript elongation (Dimitrova et al., 2008) and correlated with changes in rDNA methylation (Papazova et al., 2001; Dimitrova, 2011).

The main objective of this work was to investigate the methylation pattern of rRNA genes in two reconstructed barley karyotypes (*Hordeum vulgare* L.) with altered position or structure of the two nucleolus organizers (NORs). In addition, the method of molecular hybridization with defined rDNA probes allowed us to analyze the length and structural organization of IGS in the two different rDNA repeat units in barley.

MATERIALS AND METHODS

Plant materials and growth conditions

Homozygous structural mutants produced by γ-irradiation of dry seeds from standard variety Freya were used throughout the experiment. The control line T-1586 contained reciprocal translocation including the short arm of chromosome 3H and the long arm of chromosome 4H (Gecheff, 1996). This line served as an initial source for obtaining NOR6H-deletion karyotype T-35 (Gecheff et al., 1994) and karyotype T-21 which contained NOR6H split into two approximately equal parts, the distal one being transferred to the short arm of the chromosome 5H (Gecheff, 1989). Dry seeds from the investigated lines were soaked for 1 h in tap water and germinated for 5 days at 25°C in the dark. Shoots were used as plant material throughout the experiments.

Genomic DNA extraction, restriction analysis and southern hybridization

DNA was isolated using the conventional phenol extraction method. Restriction enzyme analysis was carried out according to supplier's instructions (FERMENTAS, Lithuania).

Molecular hybridization with different DIG-labeled rDNA probes was performed using DIG DNA Labeling and Detection Kit (Roche) following the manufacturer's protocol. DNA probes used in the molecular hybridization experiments (Fig. 1) were subcloned in pBR322 plasmid vector by Hvarleva et al. (1987) in the laboratory of Prof E. V. Ananiev (Moscow, Russia). They were kindly provided by Dr. Hvarleva. R10 clone is 10 kb long EcoRI-EcoRI fragment representing the long rDNA repeat unit in barley. R3.2 represents a 3.2 kb EcoRI-Bam HI DNA fragment including the 3′-end of 25S rRNA gene and a variable part of IGS. R1.8 contains 1.8 kb Bam HI-Bam HI DNA fragment which spans the 3′-end of IGS and part of the 5′-end of 18S rRNA gene. R3.8 contains 3.8 kb Bam HI- Bam HI DNA fragment covering structural 18S-25S rRNA genes. R0.7 represents 0.7 kb TaqI – TaqI fragment spanning the 3′-end of 25S rRNA gene and 5′-IGS region of barley rDNA.

Computer programme

The structure of IGS was determined based on the published in the database sequences of the long (GenBank HQ825319) and short (GenBank AF1475501) rDNA repeat units in barley using Clone manager programme.

Methylation assay

Double digests of purified genomic DNA were performed with the restriction enzymes EcoRI and HhaI (1µg DNA with 3 – 5 units of enzyme) according to the supplier's instructions (FERMENTAS). Following electrophoretical separation and blotting the generated restriction fragments were hybridized with a barley 0.7 kb TaqI - TaqI rDNA probe labeled with [α ^{32}P] dCTP using Megaprime DNA Labeling System (Amersham).

Hybridization of the membranes was carried out at 42°C in 5x SSPE, 5x Denhardt, 1% SDS, 40% formamide and 100 µg/ml sonicated fish sperm DNA. Filters were washed 2 times each in 2x SSPE, 0.1% SDS; in 1x SSPE, 0.1% SDS and in 2x SSPE at 65°C. Membranes were dried and exposed to Kodak MXG films for 3 – 72 h at –30°C.

RESULTS AND DISCUSSION

Variation in the length of IGS in rDNA has been described for many plant species including barley (Appels and Dvorak, 1982; Allard et al. 1990; Pillay, 1996; Warpeha et al. 1998; Fernandez et al., 2000; Abdulova and Ananiev, 2003; Carvalho et al., 2011; Grabiele, 2011). To determine the length of IGS in the control barley karyotype T-1586, a single or double digestion of genome DNA with EcoRV and EcoRI was carried out followed by Southern hybridization with several DIG-labeled probes.

As mentioned earlier, the restriction endonuclease EcoRI differentiates two main rDNA repeat units in barley each with a length of 8.8 kb and 9.8 kb, respectively. On the other hand, studies of Saghai-Maroof et al. (1984) and Kumar and Subrahmanyam (1999) showed that restriction endonuclease EcoRV generated three fragments with a size of 7.0 kb, 6.1 kb and 2.9 kb, respectively. Clone Hv014 contains the entire long rDNA repeat unit (designed as R10) inserted into the plasmid pBR325 at its single EcoRI site (Ananiev et al., 1986). Complete restriction of R10 with BamHI results in generation of four fragments with a length of 1.8 kb, 3.2 kb, 3.8 kb and 1.0 kb, respectively (Fig 1). It is well known that subclones R1.8 and R3.2 contain mainly regions of IGS

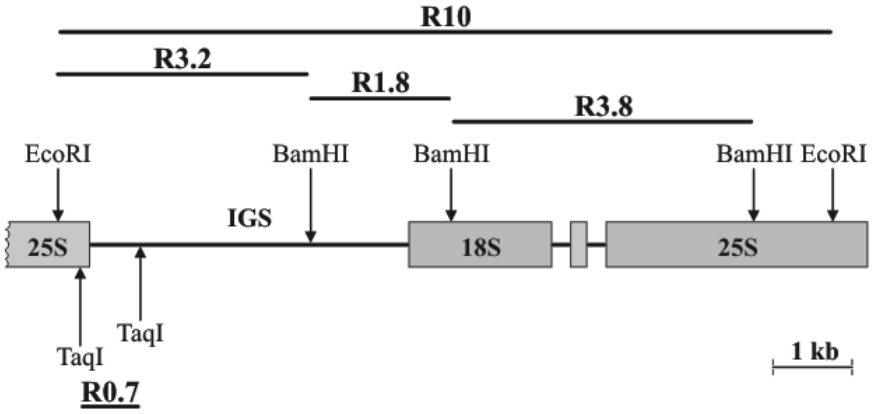

Figure 1. Restrction map of the long barley rDNA repeat unit. Positions of EcoRI, BamHI and TaqI restriction sites are indicated with arrows. The structural gene for 5.8S rRNA is presented with a small unmarked rectangle. IGS – intergenic spacer.

whereas subclones R3.8 and R1.0 contain parts of the coding regions of the rRNA genes (Hvarleva et al., 1987).

The insert R10 was separated from the plasmid after digestion with EcoRI. The isolated DNA fragment with a length of 9.8 kb was further fragmented with the restriction endonuclease EcoRV. Genome DNA from the control barley karyotype T-1586 was digested either with EcoRV or EcoRI/EcoRV. The obtained DNA restriction fragments were separated in 0.8% agarose gel and transferred to membrane Hybond. Hybridization was carried out with digoxigenin (DIG-UTP) labeled probes R10, R3.2, R1.8 and R3.8.

The complete digestion of R10 insert with EcoRV followed by hybridization with R10 resulted in the generation of three fragments with a size of 3.7 kb, 3.2 kb and 2.9 kb, respectively (Fig. 2A). The single digestion of genome DNA from T-1586 with EcoRV followed by hybridization with R10 resulted in the generation of three fragments with a size of 6.9 kb, 5.9 kb and 2.9 kb, respectively (Fig. 2A). Hybridization with the subclones R1.8, R3.2 and R3.8 showed that the first two fragments contained the entire structural gene for 25S rRNA, and the majority of IGS of the long and short repeat rDNA unit, respectively (Fig. 2B, C and D). The double digestion with EcoRI + EcoRV of genomic DNA from the control line T-1586 followed by hybridization with R10 resulted in the appearance of three fragments with a length of 3.7 kb, 2.9 kb and 2.7 kb, respectively (Fig. 2A). Hybridization with the four DIG labelled rDNA subclones listed above indicated that 3.7 kb and 2.7 kb fragments contained the entire structural gene for 25S rRNA, and the major part of IGS generated both from the long and short rDNA repeat units, respectively (Fig. 2B, C and D). Hybridization with subclones R3.2, R1.8 and R3.8 indicated that the 3.7 kb-long and 2.7 kb-long fragments mainly consisted of IGS from NOR5H and NOR6H rDNA repeat units, respectively while DNA fragments with a length of 3.2 kb and 2.9 kb contained sequences of the structural genes for 18S and 25S rRNA, respectively (Fig. 2B, C, D).

These data were used to map the EcoRV restriction sites of the rRNA genes in barley (Fig. 3). The positions of the coding regions are defined by analogy with the existing restriction maps of rRNA repeat units in barley (Appels et al., 1980; Hvarleva et al., 1987). Based on the sequence data, IGS of the longer rDNA repeats (NOR6H) is about 4000 bp in length, while IGS of the shorter rDNA repeats (NOR5H) is about 2500 bp long (GenBank HQ825319, GenBank AF1475501). Our results indicated that in the control barley line T-1586 all rRNA gene copies of NOR6H had IGS with the same length, which did not differ from the estimated length of the published in the database barley rDNA clone (GenBank HQ825319). Despite that all rRNA gene copies from NOR5H had the same IGS length, it differed with about 500 bp from that of the previously published in the database rDNA clone (GenBank AF1475501). Spacer length variants have been observed in different barley varieties and its wild relatives (Hvarleva et al., 1987; Saghai-Maroof et al., 1990). A different number of 115 bp subrepeats has been shown to be a major source of repeat length variation in barley (Saghai-Maroof et al., 1990; Molnar et al., 1992). Obviously, the short rDNA repeat unit in

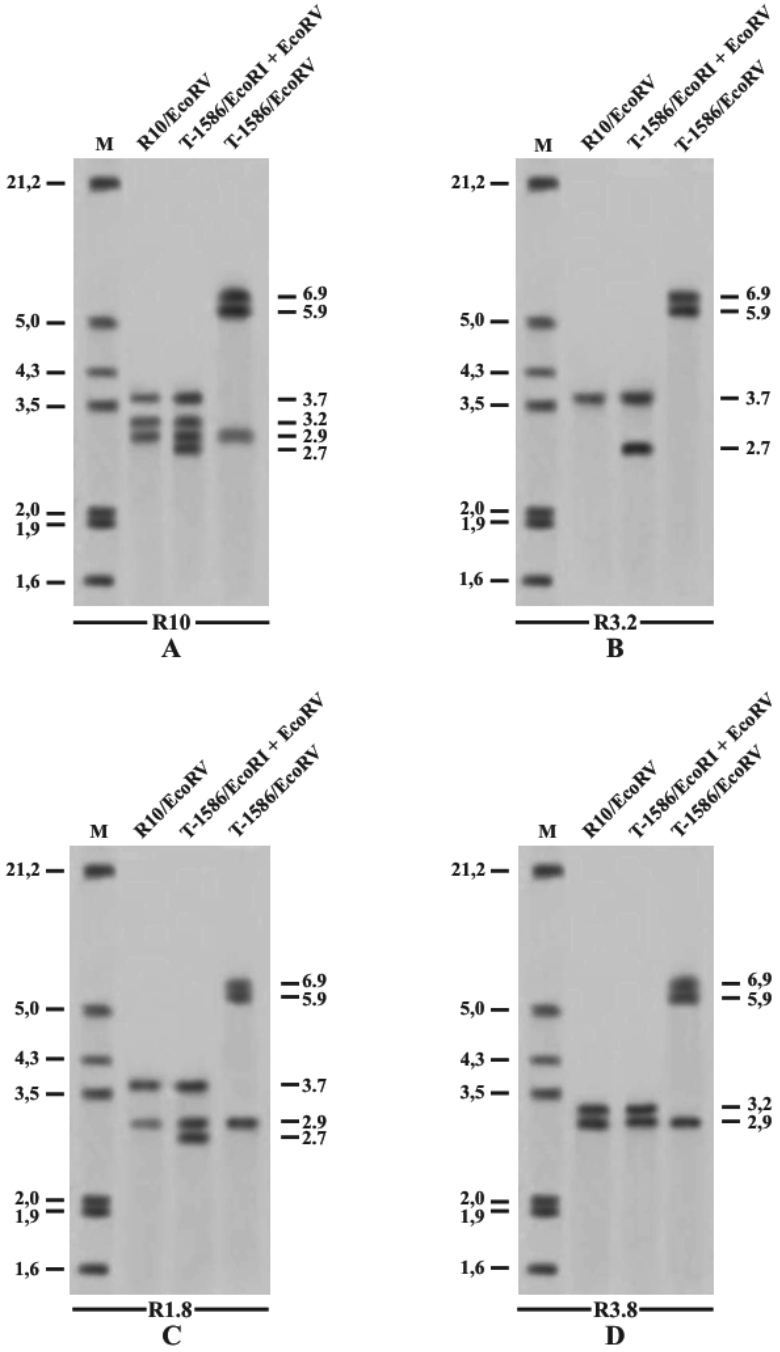

Figure 2. Hybridization banding pattern of barley rDNA. Genome DNA from T-1586 was digested with either EcoRI/EcoRV or EcoRV. Insert R10 was separated from the plasmid (pBR325) by digestion with EcoRI and was further restricted with EcoRV. Hybridization was performed with DIG-labeled probes: R10 – the long rDNA repeat unit of barley (A); R3.2 – subclone of R10 containing 3′-end of 25S rRNA gene and variable part of the IGS (B); R1.8 – subclone of R10 containing part of IGS and part of the 18S rRNA gene (C); R3.8 – subclone of R10 containing part of 18S, the whole 5.8S and part of 25S rRNA genes (D); M – marker DNA (λ-phage DNA digested with EcoRI and Hind III).

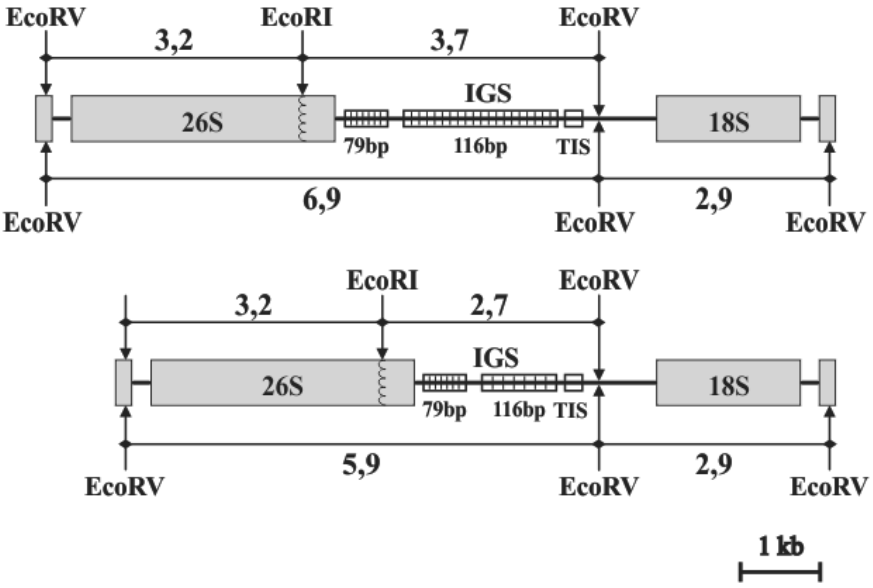

Figure 3. Restriction site map of long (A) and short (B) 18S-5.8S-25S rDNA repeat units in barley. Positions of EcoRI and EcoRV restriction sites are indicated with arrows. Regions containing 79 bp subrepeats and 116 bp subrepeats are marked. The structural genes for 5.8S rRNA are presented with a small unmarked rectangle. IGS – intergenic spacer; TIS – Transcription initiation site.

our barley lines possessed a higher number of 116 bp subrepeats as compared with the previously sequenced clone (GenBank AF1475501).

Differential expression of genes is most commonly associated with methylation of cytosine residues in DNA. Studies of epigenetic regulation in plants and mammals reveal the evolutionary conservatism of epigenetic mechanisms, including DNA methylation (Habu et al., 2001). The rRNA gene expression is affected by the methylation of regulatory sequences or enhancer elements present in the IGS region (Sharma et al., 2005). Methylation of the C residues can be best studied by methylsensitive restriction endonucleases such as HhaI. The enzyme can cut unmethylated cytosine in the short –GCGC– palindrome. If both Cs (separately or together) are methylated, the enzyme is not able to cut the sequence.

To analyze the role of methylation for rRNA gene activity in barley lines with altered position or structure of the NORs, the rDNA methylation profile in the studied variants was investigated. The methylation profiles of the control (T-1586), NOR-deletion (T-35) and translocation (T-21) karyotypes were analyzed for mapping the active HhaI sites within IGS. Bulk DNA from the analyzed lines was digested with EcoRI + Hha I. The length of the resulting fragments was determined by Southern hybridization with the ^{32}P-labelled 0.7 kb TaqI – TaqI fragment spanning the 3′-end of 26S rRNA gene and 5′-IGS region of barley rDNA (R0.7). EcoRI digest of T-1586 and T-35 DNA resulted in the appearance of bands with an expected length of 9.8 kb and 8.8 kb in the control and 8.8 kb in the NOR-deletion line (Fig. 4). Double digestion of EcoRI + HhaI showed that the majority

of rDNA remained undigested in a high molecular weight fraction. This result indicated a high level of methylation in the –GCGC– recognition sites in the three lines. Although a large amount of rDNA remained undigested, fragments with a smaller size (between 0.2 kb - 7.2 kb in T1586 and 0.2 kb - 6.3 kb in T-35) were detected on the autoradiographs. The methylation profile of T-21 did not differ from that of the control line in number and intensity of the bands (Fig. 4). Fragments with a size of 0.5 kb, 0.6 kb and 0.7 kb were localized into the 79 bp subrepeats of the both repeat units (Fig. 5). 0.4 kb, 0.5 kb and 0.6 kb fragments hybridized more intensively in T-35 compared to T-1586 indicating that more rDNA repeats in the deletion line had hypomethylated -GCGC- sites. Fragments with a length of 1.1 kb, 1.3 kb, 1.9 kb, 2.2 kb and 3.9 kb were derived from the IGS of NOR6H as they were not present in T-35. Fragments with a length of 2.4 kb, 2.6 kb and 2.9 kb were observed in all three lines, thus showing that they were generated by HhaI recognition sites in the IGS of the short rDNA repeat unit. Since 2.6 kb and 2.9 kb bands represented the most intensive hybridization signals in the NOR-deletion karyotype it could be assumed that most probably in most of the rDNA repeats these sites were demethylated. Fragments with a length between 4.4 kb and 7.3 kb were generated by HhaI restriction sites in the structural genes for 18S-5.8S-25S rRNA. A fragment with a size of 3.4 kb was detected in the hybridization profile of T-35, but it was absent in T-1586 and T-21. This fragment was localized in the structural gene for 18S rRNA (Fig. 5).

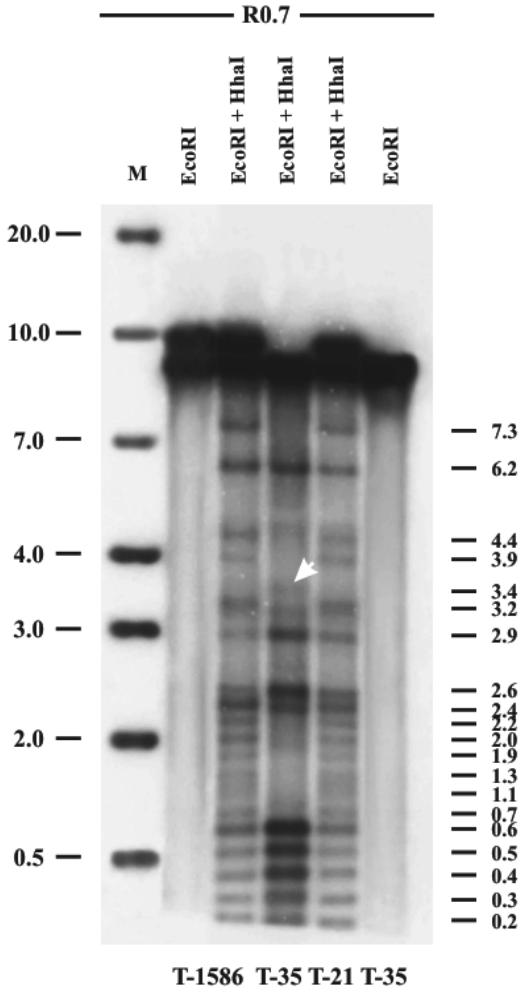

Figure 4. Methylation pattern of barley rDNA. Genome DNA was completely digested with EcoRI + HhaI. Hybridization was carried out with ^{32}P-labelled 0.7 kb TaqI–TaqI DNA fragment spanning the 3′-end of 25S rRNA gene and 5′-IGS region of barley rDNA. The lengths of the generated fragments (kb) are shown on the right side of the autoradiography. M – marker DNA (1 kb DNA ladder, FERMENTAS). The newly generated hypomethylated DNA fragment in the NOR-delition line T35 is indicated by an arrowhead.

The positions of the putative HhaI sites in both IGS as revealed from the primary structure published in the database are indicated in Fig. 5. Based on the conservatism of the structural rRNA genes the positions of the possible HhaI

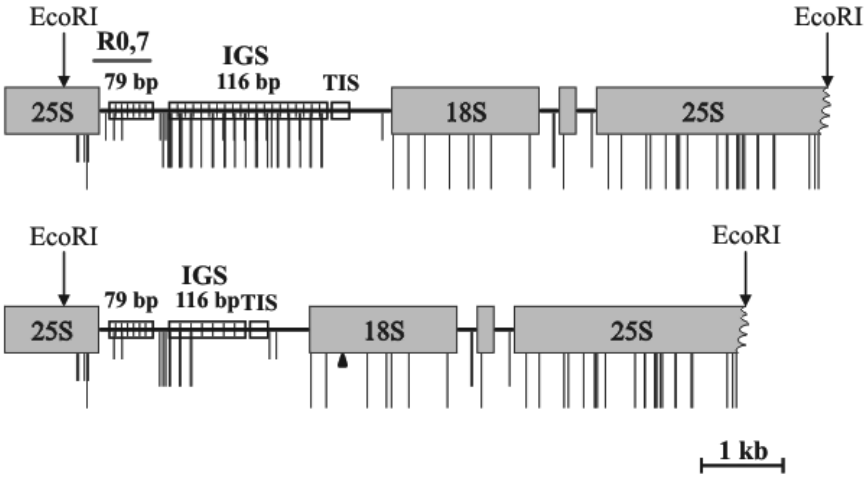

Figure 5. HhaI map of rDNA repeat units in barley. The length of EcoRI-generated restriction fragments and the positions of HhaI sites in IGS and structural 18S-25S rRNA genes are indicated. The structural genes for 5.8S rRNA are presented with a small unmarked rectangle. DNA probe used in the hybridization analysis is marked by R0,7. Regions containing 79 bp subrepeats and 116 bp subrepeats in IGS are presented. IGS – intergenic spacer; TIS – Transcription initiation site. Demethylated HhaI sites in IGS are indicated by short vertical lines. The newly generated hypomethylated HhaI restriction site is indicated by an arrowhead.

sites in the 18S-25S rRNA genes are also shown. As deduced from autoradiographs, active HhaI sites were located in the 79 bp subrepeats, 116 bp subrepeats and the external transcribed spacer (ETS) (Fig. 5).

Our results showed that the differential expression of the NORs in the translocation line T-21 did not correlate with changes in the methylation of –GCGC– neither in IGS nor in the coding regions. These data confirmed the conclusions of Papazova et al. (2001) regarding the limited role of methylation in the case of intraspecific nucleolar dominance.

In the NOR-deletion line T-35, the increased activity of ribosomal RNA genes in chromosome 5H correlated with hypomethylation of -GCGC- sites localized in the IGS. The results obtained by Ruffini et al. (2010) on the distribution of 5-methylcytosine along the chromosomes showed that deletion of NOR6H in karyotype T-35 led to a lower level of DNA methylation in the satellite 5H chromosome. Our results were in agreement with this observation.

Methylation pattern of rRNA genes in barley is complex. Most of repeated rDNA units are resistant to Hha I digestion, indicating that they are methylated in all-GCGC-sites located both in IGS and in the structural genes for 18S - 25S rRNA. Our results allowed to assume that a small fraction of the existing HhaI sites in repeated rDNA units were hypomethylated.

IGS region of rRNA genes is a complex unit, which includes rDNA transcription regulatory elements being very important for their expression (Polanco and Pérez De La Vega, 1997). Sometimes the regulatory elements are included in the subrepeats, and it has been indicated that not only the subrepeat types, but also their

numbers and their methylation levels are important for transcription (Polanco and Pérez De La Vega, 1997). Several studies revealed a close correlation between the IGS length variability and the cytosine methylation status in wheat (Flavell et al. 1988; Sardana et al. 1993; Carvalho et al. 2010).

In conclusion, our results indicate that the compensatory effect in the expression of rRNA genes in T-35 observed earlier (Dimitrova et al., 2008) was accompanied by increased hypomethylation of the rRNA genes in the only remaining NOR5H.

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