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Characterization of relic DNA from barley genome

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Summary. High-molecular-weight "relic" DNA fraction can be electrophoretically separated from the bulk of barley DNA digested with different restriction enzymes. We have cloned and analyzed a population of relic DNA fragments. The majority of AluI-relic DNA clones contained barley simple sequence satellite DNA and other families of repetitive DNA. One of these families, designated HvRT, has been analyzed in detail. This family is composed of tandemly arranged 118-bp monomers and is present in 7×10^5 copies in the barley genome. Clones representing the HvRT family were sequenced. HvRT repeats were found to contain high levels of methylated cytosine. The HvRT family was found in the genomes of *H. vulgare, H. leporinum, H. murinum, H. jubatum,* but not in *H. marinum, H. geniculatum,* and wheat. Different barley species and cultivars show restriction fragment length polymorphism with the HvRT probe. Chromosome-specific subfamilies of HvRT were found to be present on different barley chromosomes, providing the possibility of using the HvRT probe as a chromosome-specific marker. HvRT fragments up to 810 kbp in length were resolved by pulsed field gel electrophoresis.

Key words: Chromosome marker – *Hordeum vulgare* – Relic DNA – Tandemly repeated sequences

Introduction

Repetitive DNA sequences constitute up to 80% of higher plant DNA (Flavell 1982). Some are scattered in the genome, whereas others are organized as clusters of tandemly repeated units. Sequences of both types were found in different higher plant species including barley (Ananiev et al. 1988). Tandemly repeated DNA sequences (TRS) were characterized in detail at the molecular level in rye (Bedbrook et al. 1980), maize (Dennis and Peacock 1984), rice (Wu and Wu 1987), melon (Leclerc and Siegel 1987), onion (Barnes et al. 1985), broad bean (Kato et al. 1984), *Arabidopsis* (Martinez-Zapater et al. 1986), and some other *Cruciferae* (Benslimane et al. 1986; Grellet et al. 1986). They share some features in common: (1) tandem organization in the genome, (2) high copy number, (3) incomplete identity of monomeric units, (4) similar length of these units (usually 170–360 bp), (5) high level of cytosine methylation, (6) absence of transcriptional activity, and (7) predominant localization of clusters in the centromeric or telomeric regions of most, if not all, chromosomes. Some TRS families may not have all these features simultaneously. As was shown earlier (Bedbrook et al. 1980), plant genomic DNA fraction enriched with TRS can be obtained in the form of relic DNA resistant to the action of restriction enzymes. This relic DNA may be composed of different either related or unrelated sequence families and represents a good source for species-specific probes (Metzlaff et al. 1986; Junghans and Metzlaff 1988), which are used as molecular genetic markers. Moreover, such probes can be applied as cytogenetic markers in combination with in situ hybridization techniques.

Materials and methods

Seeds of different barley cultivars were obtained from Dr. A. Pomortzev (Institute of General Genetics, Moscow). Barley-wheat addition lines as well as parental forms were kindly provided by Dr. A.K.M.R. Islam (CSIRO, Australia). Total DNA was isolated from 5-day-old barley seedlings according to Della-

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porta et al. (1983). Relic DNA for cloning was purified by centrifugation of the restriction enzyme-digested total barley DNA in NaCl gradient (Grosveld et al. 1982). Partial fragmentation of barley relic DNA was done using DNaseI in the presence of Mn^{2+} ions (Anderson 1981). Phagemid vector pBS(+) and *E. coli* recipient strain XL-1 Blue (Stratagene) were used for cloning. Plasmid DNA was isolated using the rapid alkaline extraction method (Kieser 1984). The alkaline procedure was used for DNA blotting (Reed and Mann 1985). DNA was sequenced by the dideoxy-method (Sanger et al. 1977) either after recloning the inserts into M13tg131 (Kieny et al. 1983) or using the single-stranded form of the recombinant phagemid as a template, produced in the presence of the M13K07 helper phage. All other DNA manipulations were performed following standard protocols (Maniatis et al. 1982). Pulsed field gel electrophoresis (Schwartz and Cantor 1984) was performed using an LKB Pulsaphor apparatus. DNA sequences were analyzed using the SEQBUS software for sequence analysis (Institute for Molecular Genetics, Moscow) and the repetitive DNA sequences data base constructed by Dr. N.V. Milshina in our laboratory.

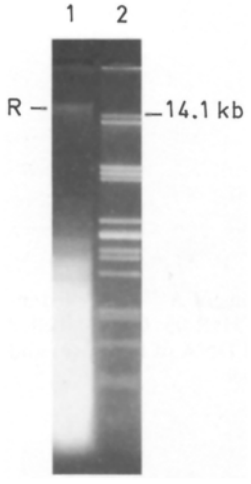

Fig. 1. Electrophoretic pattern of total barley DNA digested with AluI restriction endonuclease (*lane 1*). λ DNA digested with PstI was used for size markers (*lane 2*). Relic DNA is indicated on the *left* (*R*)

Results

Characterization of barley AluI-relic DNA using restriction endonucleases

High-molecular-weight "relic" DNA fraction can be observed after electrophoretic separation of total barley DNA digested with different restriction enzymes. This fraction consists of the mixture of extremely long restriction fragments, which most probably are built up from tandemly arranged monomeric units lacking particular restriction enzyme sites, or having these sites methylated and thus inaccessible to cleavage. AluI-generated relic DNA was chosen for further analysis because this particular fraction can be efficiently separated from the bulk of hydrolyzed DNA (Fig. 1). All restriction enzyme digestions were carried out in the presence of λ phage DNA as internal control to confirm the completeness of digestion.

We found that AluI-relic DNA cannot be completely converted into low-molecular-weight fragments by digestion of either total barley DNA or isolated AluI-relic DNA with restriction enzymes EcoRI, BamHI, BglII, HindIII, HinfI, AluI, Sau3A, MspI, BspRI, TaqI, and all of their possible pairwise combinations. However, when different restriction enzyme digests of barley DNA were blot-hybridized with AluI-relic DNA as a probe, a set of fragments of low molecular weight as well as the band of relic DNA were observed (Fig. 2A). At least two different ladders consisting of regularly spaced bands were discerned (e.g., in EcoRI and Sau3A digests) against the background of a rather complex hybridization spectrum. This pattern is typical of tandemly organized repetitive

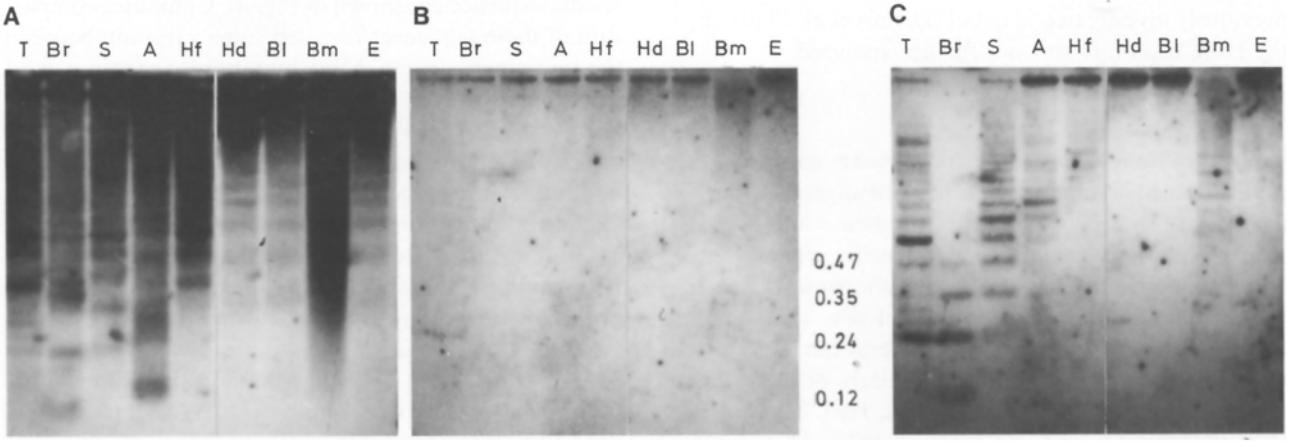

Fig. 2. Hybridizations of **A** ^{32}P-labelled AluI-relic DNA; **B** ^{32}P-labelled insert of HvR 11; **C** ^{32}P-labelled HvRT probe to Southern blots of total barley DNA digested with various restriction endonucleases and separated by electrophoresis on 2% agarose gel. *T*-TaqI; *Br*-BspRI; *S*-Sau3A; *A*-AluI; *Hf*-HinfI; *Hd*-HindIII; *Bl*-BglII; *Bm*-BamHI; *E*-EcoRI. The size of 118-bp oligomers is indicated in kb (**C**, left margin)

sequence families (Horz and Zachau 1977). Thus, these experiments show that: (1) AluI-relic DNA is composed of different repeated DNA sequence families, (2) some of these families are tandemly repetitive, and (3) there is some component in AluI-relic DNA lacking the sites for all ten restriction enzymes used.

Cloning of AluI-relic DNA

AluI-relic DNA was cloned in two ways. First, total barley DNA was completely digested with TaqI and subsequently ligated into the AccI site of the vector. However, preliminary experiments showed that AluI-relic DNA cannot be totally cleaved in this way. In order to get a more representative library of relic DNA, this fraction was subjected to limited cleavage with DNaseI in the presence of Mn^{2+} ions, to result in an average fragment size of 500 bp, with subsequent end repair using T4 DNA polymerase and ligation into the SmaI site of the vector. Both libraries were screened by the colony hybridization procedure using nick-translated AluI-relic as a probe. Approximately 1,300 clones from each library were probed, and 6 "TaqI-generated" and 22 "DNaseI-generated" clones were selected for further characterization. Insert size varied from 60 to 1,000 bp, with the most abundant class being aproximately 160–250 bp. Cross-hybridization with the labelled inserts of some clones obtained showed that most of them belong to two families (Table 1).

When the HvR 11 insert was hybridized to the Southern blot of different restriction digests of barley DNA, it revealed only the band of relic DNA in all digests (Fig. 2B). The most probable explanation of such a hybridization pattern is that the HvR 11 family consists of the fragments of barley cryptic satellite DNA. This point was further confirmed by partial sequencing of the HvR 05 clone insert, which is also a member of the HvR 11 family (Table 1). This cryptic satellite DNA has been previously investigated in detail (Dennis et al. 1980), thus the HvR 11 family was not further analyzed.

HvRT family analysis

Several randomly selected clones were used for hybridization to Southern blots of AluI digests of barley and wheat DNA in order to confirm their relationship to relic DNA and to select species-specific clones. Three autoradiograms from this series are shown in Fig. 3. Indeed, these clones hybridized to AluI-relic and some of them demonstrated, in addition, several bands of lower molecular weight spaced either regularly or irregularly. Five clones tested (HvT 01, HvT 02, HvT 03, HvT 04, HvR 06) were found to be barley-specific, i.e., they did not show any hybridization to wheat DNA even under nonstringent conditions (hybridization at 65 °C in 5 × SSC, washing at 65 °C in 2 × SSC). We selected for further analysis the barley-specific HvRT family, represented by clones HvT 01, HvT 02, and HvR 06.

Table 1. Classification of the AluI-relic DNA-derived clones into families according to the cross-hybridization results

Family designation	Probe	Hybridizing clones
HvRT	HvR 06	HvR 06, HvT 01, HvT 02,
HvR 11	HvR 11	HvR 06, HvR 02, HvR 04, HvR 05, HvR 07–HvR 11, HvR 15–HvR 17
HvT 03	HvT 03	HvT 03

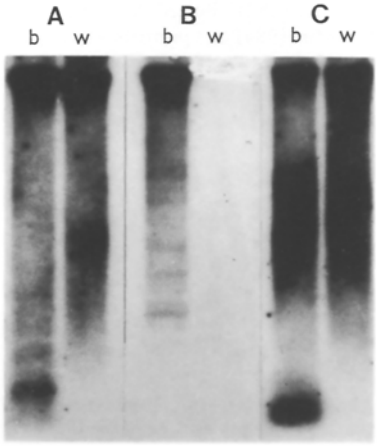

Fig. 3A–C. Southern blot hybridizations of **A** ^{32}P-labelled insert of HvR 05; **B** ^{32}P-labelled insert of HvR 06; **C** ^{32}P-labelled insert of HvR 08 to AluI-digested total DNA of barley (*b*) and wheat (*w*) separated in 0.8% agarose gel

Nucleotide sequence determination of these clones demonstrated that the HvRT family consists of tandemly repeated units 118 bp long (primary structures and consensus sequence are shown in Fig. 4). Computer comparison of these sequences revealed some variation between the monomers due to point mutations, insertions, and deletions. However, their average homology to the consensus is high (>94%). A search for the nucleotide homology to known repeated DNA sequences of different origin, including recently published plant satellite DNA sequences and those contained in GENBANK (release 54), did not show any significant homologies.

Genomic Southern blots performed with the HvRT probe to different restriction enzyme digests of barley DNA revealed typical ladder patterns in HinfI, AluI, Sau3A, BspRI, and TaqI digests, indicating tandem arrangement of the HvRT family (Fig. 2C). In particular, BspRI totally cleaved this family to mono-, di-, tri- and tetramer [type A pattern according to Horz and Zachau (1977)], while four other enzymes produced relic DNA and ladders starting from the dimer. BamH1 produces

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HVT01   1-tcgaaactcgcatttttggcctattctggctagttctgcatgctattgctcactgatttt- 60
HVT01 119-..a.....................c.cca..c.a..t..t........ag...a.......-178
HVT02   1-..g......a..g......t.....tt...c....t..t........a....t....a..- 60
HVT02 119-.ga.........g............tt...c....t..t........a.......c....-178
HVR06   1-                                                  ........-  8
HVR06  68-..a.......t.g....a.......cc...c....t..t..c.....ctggcta.ga...-127
HVRT    1-TCRAAACTCGCAGTTTTGGCCTATTYYGGCCAGTTTTGTATGCTATTRWTCAETGATTTT- 60

HVT01  61-ggatcccgctgcgatccgaacgttttggggaaccccgggggccggttacg-ggaactct-118
HVT01 179-g.g.......at..a..a........-c....                        -208
HVT02  61-t.g.......gg.....g...........-.c........t...a.....t.......a-118
HVT02 179-g.t.g.....aa.....a........-g....a........t.........g.......g-236
HVR06   9-g.g.t...a.gt.....g........-c....c...a....t.........g.......a- 67
HVR06 128-tacg.ttag.cact-------....tag                             -148
HVRT   61-LGGTCCCGCTRNGATCCRAACGTTTTWGGGAECCCCGGGGLCCGRTTACGLGGAACTCN-119
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Fig. 4. Nucleotide sequences of the clones representing the HvRT family. All sequences are divided into two parts to illustrate the conservation of the DNA sequence between the monomers. Only the positions of nonhomology are indicated for HvT 02, HvR 06, and the second (truncated) monomer of HvT 01. Consensus sequence is also shown (*lower lane*)

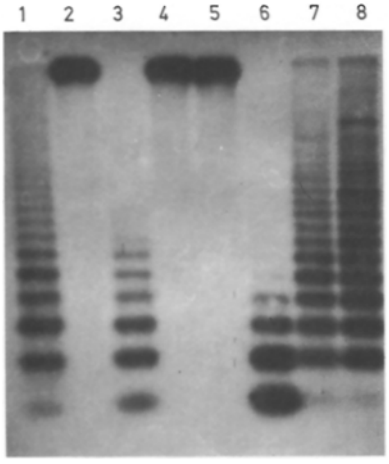

Fig. 5. Cleavage of the HvRT family with methylation-sensitive restriction enzymes as revealed by Southern hybridization with ^{32}P-labelled HvRT probe. *Lanes 1–7*: barley DNA, cultivar Donetzky 4, cleaved with MspI (*1*), HpaII (*2*), MvaI (*3*), EcoRII (*4*), AtuBI (*5*), MboI (*6*), Sau3A (*7*). *Lane 8*: barley DNA, cultivar Odessky 9, cleaved with Sau3A

Table 2. Restriction enzymes used for HvRT sequence methylation assay, their recognition sequences, and methylation sensitivity

Restriction enzyme	Cleavable site	Uncleavable site
MspI	CCGG, C^{m}CGG	^{m}CCGG
HpaII	CCGG	^{m}CCGG, C^{m}CGG
EcoRII	CCA/TGG	C^{m}CA/TGG
AtuBI	CCA/TGG	C^{m}CATGG
MvaI	CCA/TGG, C^{m}CA/TGG	–
MboI	GATC, GAT^{m}C	–
Sau3A	GATC	GAT^{m}C

the series of larger oligomers corresponding to 5, 6, 7, 9, and 11 monomers. Finally, EcoRI, HindIII, and BglII sites are rare in HvRT clusters, since they produce no ladders.

Estimation of the copy number of the HvRT monomer in the barley genome was performed by quantitative dot hybridization and found to be approximately 7×10^5 (not shown). Thus the HvRT family comprises about 1.7% of barley DNA, corresponding to the total length of HvRT clusters, ca. 90,000 kbp.

Blot hybridization of the HvRT probe with the Southern blots of total barley DNA digested with different restriction enzymes and their methylation-sensitive isoschizomers (Table 2, Fig. 5) allowed the following conclusions to be drawn. First, comparison of MspI to HpaII patterns, as well as MvaI to EcoRII and AtuBI patterns, shows that cytosine residues are highly methylated in CG, CAG, and CTG sites in HvRT clusters. At the same time, external cytosines in CCG triplets are methylated to a lesser extent, since MspI produces a characteristic ladder rather than a high-molecular-weight (relic) band (Fig. 5, lane1). Comparison of MboI and Sau3A patterns shows that all bands larger than pentamer in the Sau3A pattern are due to methylation of 3′-cytosine in the GATC sites. Moreover, Sau3A patterns of two barley cultivars (Donetzky 4 and Odessky 9) differ in the presence of "amplified" bands of different sizes (Fig. 5, lanes 7 and 8).

The distribution of the HvRT family in the genus *Hordeum* was studied by Southern hybridizations to BamHI-digested DNAs of several barley species under different stringency conditions ($3 \times$ SSC and 30%, 40%, or 50% formamide at 48 °C). HvRT homologous families were detected in the genomes of *H. violaceum, H. murinum, H. jubatum,* and *H. leporinum,* but not in the genomes of *H. marinum, H. geniculatum,* and wheat (Fig. 6). This finding is in accordance with recent phylogenetic studies (Jorgensen 1986). In addition, restriction

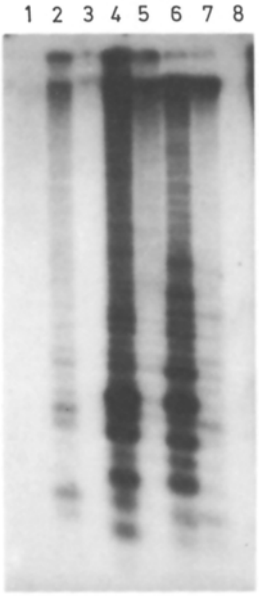

Fig. 6. Southern blot hybridization of the HvRT probe to BamHI-digested total DNA of *Hordeum marinum* (*1*); *H. murinum* (*2*); *H. geniculatum* (*3*); *H. violaceum* (*4*); *H. jubatum* (*5*); *H. vulgare* (*6*); *H. leporinum* (*7*); *T. aestivum* (*8*). All lanes contained 4μg DNA

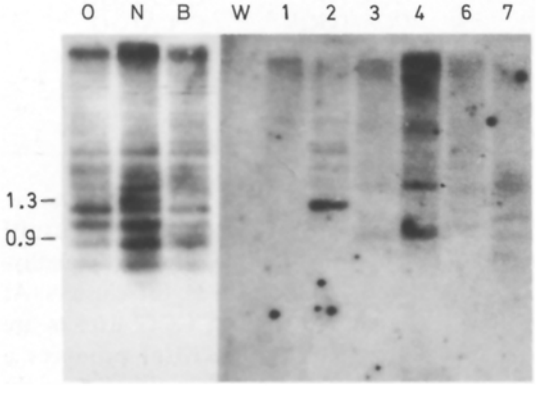

Fig. 7. Southern blot hybridization to BamHI-cut DNA from barley, wheat, and barley-wheat chromosome addition lines (AD): *O*–barley cv Odessky 9; *N*–barley cv Nutans 45; *B*–barley cv Betzes; *W*–wheat cv Chinese spring; *1*–AD1; *2*–AD2: *3*–AD3; *4*–AD4; *6*–AD6; *7*–AD7. ^{32}P-labelled HvRT sequence was used as a probe. All lanes contained 3 μg DNA, except AD4 lane (8 μg)

fragment length polymorphism can be observed between species, which is more pronounced in the high-molecular-weight range. Variation in the hybridization stringency did not result in qualitative changes of hybridization patterns (not shown). This suggests that the homology of the HvRT-related families from different barley species is relatively high.

Blot hybridization of the HvRT probe with BamHI digests of different barley cultivars (Fig. 7) also showed restriction fragment length polymorphism. A band of

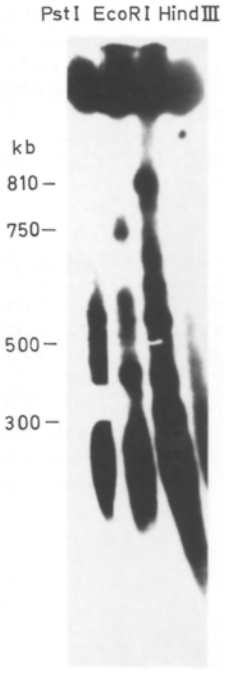

Fig. 8. Southern blot hybridization of the ^{32}P-HvRT probe to PFGE-separated total barley DNA digested with PstI, EcoRI, and HindIII

0.9 kb was detected in the Betzes cultivar, but was absent from Nutans 45 and Odessky 9, whereas the 1.3-kb band was characteristic of Nutans 45 only. Many different cultivars of barley were screened in this way, and it was found that the hybridization patterns of the related cultivars are identical (not shown).

To investigate the localization of HvRT family sequences on barley chromosomes, we used Southern blot hybridization on a panel of BamHI-digested DNAs from barley-wheat addition lines (Islam et al. 1981). Barley chromosomes 2, 3, 6, and 7 appear to possess chromosome-specific hybridization patterns, while chromosomes 3 and 4 showed similar patterns (Fig. 7). The addition line containing chromosome 5 was not obtained, and DNA from addition line 1 did not show clear results. Each individual band could be traced back to its particular counterpart in the digest of barley, cv Betzes, which was used as a source of barley chromosomes for the production of the addition lines. The conclusion made from this experiment is that barley chromosomes 2, 3, 6, and 7 bear chromosome-specific HvRT clusters.

To estimate the upper size limit of HvRT clusters, pulsed field gel electrophoresis (PFGE) was employed (Schwartz and Cantor 1984). This technique allowed the fraction appearing as a high-molecular-weight relic upon conventional gel electrophoresis to be resolved into a number of bands of high molecular weight (Fig. 8). In particular, the largest HindIII fragment was approximately 810 kbp in length.

Discussion

We report here the characterization of the relic DNA from barley genome that is produced after cleavage with AluI restriction endonuclease, as well as the molecular description of one component of AluI-relic DNA – the HvRT family of tandemly arranged repeated sequences.

It can be inferred from the results of AluI-relic restriction analysis and molecular cloning that this fraction is heterogeneous. In particular, it contains cryptic satellite DNA, the HvRT family of tandemly arranged repeated sequences, as well as one other family with a similar organization pattern but having longer periodicity (Fig. 2A, lanes Hd, Bl, E) and, as has been shown earlier, the family of tandemly arranged genes coding for 5s RNA (Khvyrleva et al. 1988).

Relic DNA-derived clones containing fragments of the cryptic satellite DNA with simple sequence $(GAA)_m(GAG)_n$ were found to constitute the major part of the clones obtained using DNaseI treatment. The unusual sequence appears to be responsible for the absence of satellite DNA from TaqI-produced clones, despite the relatively high proportion of this satellite in the barley genome – about 4% (Dennis et al. 1980). The existence of an AluI-relic DNA subfraction that is inaccessible to cleavage with ten restriction enzymes, checked both individually and in all possible pairwise combinations, can be explained in the same way.

Three of five species-specific clones selected turned out to be members of the family of tandemly repeated monomeric units 118 nucleotides long (HvRT family). This family is present in about 7×10^5 copies in the barley genome. High copy number together with apparent species-specifity indicates that amplification, giving rise to the HvRT-family, has taken place relatively recently in evolution, and at least since the divergence between the *Hordeum* and *Triticum* genera and separation of the *marinum* group.

Comparison of the nucleotide sequences of HvT 01, HvT 02, and HvR 06 demonstrated that they are highly homologous to the consensus sequence – 94.4%, 97.9%, and 95.0%, respectively. Similar divergence values were observed for *Arabidopsis thaliana* (Martinez-Zapater et al. 1986), *Allium cepa* (Barnes et al. 1985), *Brassica oleracea* (Benslimane et al. 1986), and maize (Dennis and Peacock 1984) tandemly repeated sequence families. Homologies between the HvRT sequence and other known TRS found upon computer search did not exceed 50 nucleotides and were not perfect.

Analysis of the distribution of methylated cytosines in HvRT clusters demonstrated that external C in the CCG triplet is methylated to a lesser extent than in CAG and CTG triplets (Fig. 5). This result is at least in qualitative accordance with the data of Gruenbaum et al. (1981) obtained from the analysis of methylation of total wheat DNA. However, intercultivar polymorphism of the methylation pattern (Fig. 5, lanes 7 and 8) needs further study for an appropriate explanation.

An uneven distribution of the hybridization signal between oligomers of different length in TaqI, Sau3A, AluI, and HinfI digests (hybridization is observed with relic DNA and small oligomers, whereas it is absent in the case of long oligomers) allows one to propose the existence of separate domains in the HvRT family, which differ in the particular restriction enzyme site density. Furthermore, some prominent bands in TaqI (5 and 11 monomers), AluI (7 and 11 monomers), and HinfI (10 and 12 monomers) digests (Fig. 2B) may provide an indication of the amplification and evolution of the units that are longer than a 118-bp monomer. Such higher order repeat units are also seen in BamHI digests, and restriction fragment length polymorphism between different species and cultivars is also detected at this level. Moreover, higher order repeat units of different lengths proved to be localized on different barley chromosomes (Fig. 7), as happened in case of alphoid DNA in humans (Willard and Waye 1987).

Finally, pulsed field gel electrophoresis (PFGE) results allowed us to conclude that the HvRT family consists of extremely long clusters. In particular, we were able to detect a HindIII fragment of 810 kbp. It is important to note that the total length of HvRT sequences in the barley genome (calculated from dot hybridization to be 90,000 kbp) essentially exceeds the total length of hybridized fragments; i.e., all "superstructures" revealed by PFGE are themselves repetitive. Thus, the HvRT family consists of hierarchically arranged repetitive structures: (1) 118-bp monomers, (2) a set of chromosome-specific oligomers revealed by BamHI, (3) superstructures resolved by PFGE.

Thus, the HvRT probe can be used as a chromosome-specific marker for the mapping and investigation of the barley genome structure and evolution, as well as for analyses of hybrids in breeding programs.

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