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Cloning and mapping of telomere-associated sequences from *Hordeum vulgare* L.

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Summary. We present a novel approach to the efficient cloning of telomere-associated sequences and demonstrate its application to the cloning and mapping of these sequences from barley. The method is a modification of the Vectorette PCR technique and allows specific amplification and cloning of subtelomeric sequences. Telomere-associated sequences isolated from barley include hypervariable, repetitive sequences. Polymorphisms detected by subtelomeric markers behaved as Mendelian factors and were mapped to the most distal positions of barley chromosomes 1, 2, and 4.

Key words: Telomeres – Cloning – Mapping – Barley – Vectorette PCR

Introduction

Eukaryotic chromosome telomeres have distinct structural and functional features and provide important landmarks for the termini of genetic and physical maps of chromosomes (Zakian 1989; Blackburn 1991a, b). Telomere-associated sequences, located directly proximal to telomeric tandem repeats, are a source of telomeric markers. Cloning and mapping of subtelomeric sequences have remained difficult in spite of extensive efforts (Cross et al. 1989; de Lange et al. 1990; Cooke et al. 1985; Weber et al. 1990). General and simple techniques directed toward cloning of these sequences are needed.

The North American Barley Genome Mapping Project (NABGMP) is a multinational initiative aimed at constructing a saturated genetic map of the barley genome (Kleinhofs 1992). Like other genome mapping projects, NABGMP encountered the problem of identifying genetic markers that define the ends of chromosomes and provide tags for the most distally located genes. In order to obtain these markers, we designed a strategy for cloning Telomere-Associated Sequences (TASs) from barley (*Hordeum vulgare* L). We adapted the Vectorette PCR method (Arnold and Hodgson 1991; Riley et al. 1990) which allows amplification of specific DNA fragments adjacent to regions of known sequence.

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Materials and methods

The telomere primer (TP) was designed to include an *Eco*RI site (underlined) for subsequent cloning. The TP sequence is 5′CCGAATTCAACCCTAAACCCTAAACCCTAAACCC3′. The vectorette primer sequence is 5′CGAATCGTAACCGTTCGTACGAGAATCCGT3′. All oligonucleotides, including the vectorette (Riley et al. 1990), were synthesized by the Laboratory of Biotechnology and Bioanalyses at Washington State University, Pullman.

Barley cv. Steptoe DNA (3 µg), digested with *Bam*HI, was ligated with 5 pmoles of annealed Vectorette. Ligation products (50 µl) were diluted to 250 µl with H_2O and 5 µl aliquots were used as template for amplification. Amplification reactions contained template, 0.1 mM dNTPs, *Taq* polymerase buffer (Promega) and 30 pmoles of telomere or vectorette primer in a 100 µl volume. The reactions were denatured for 2 min at 94° C, 2.5 units of *Taq* DNA polymerase (Promega) were added and the reactions were overlaid with mineral oil. Amplifications were carried out for 5 cycles of 1 min at 94° C, 1.5 min at 63° C and 3 min at 72° C. Then 30 pmoles of vectorette primer were added to reactions containing the telomere primer and amplifications were continued for 30 cycles of 1 min at 94° C and 3.5 min at 71° C. Amplification products (10 µl) were analyzed on 1.5% agarose gels in TAE buffer. (40 mM TRIS-acetate, 1 mM EDTA pH 8.0).

The amplification products were digested with *Bam*HI and *Eco*RI and ligated to appropriately digested vectors. In some cases specific bands were isolated by agarose gel

electrophoresis prior to ligation. The ligation products were used to transform competent *Escherichia coli* cells and colonies carrying inserts were selected using blue/white selection on X-gal/IPTG plates.

All standard DNA manipulations were as described in Sambrook et al. (1989). Double-stranded DNA sequencing was carried out as described in Murray (1989). Sequence analyses were performed using GCG Sequence Analysis Software Package (Devereux et al. 1984). Southern blot transfer, hybridization and washing conditions were as previously described (Kleinhofs et al. 1988). The polymorphisms detected by the subtelomeric probes were mapped using 150 doubled haploid lines derived from the Steptoe by Morex cross (Kleinhofs and Kilian 1992). Linkage analyses were performed using MAPMAKER (Lander et al. 1987).

Results and discussion

Barley TASs were cloned as outlined (Fig. 1) using a target-specific primer based on the sequence of *Arabidopsis thaliana* telomere repeats (TRs) (Richards and Ausubel 1988) which have been shown to cross-hybridize with barley chromosome telomeres (Schwarzacher and Heslop-Harrison 1991). Five cycles of amplification using a telomere primer (TP), followed by 30 cycles with both TP and Vectorette primer (VP), yielded products, visible as distinct bands on agarose gels (Fig. 2). The original Vectorette PCR method (Arnold and Hodgson 1991) failed to produce specific amplification products, probably due to the large genome size of barley, ca. 5.3×10^9 bp (Bennet and Smith 1976).

Fifty-four clones with inserts between 0.2 and 2 kb were isolated. Fourteen unique clones were identified by complete or "single track" sequencing and shown to have TR-like repeats at the 3′ ends of the inserts. Two of the five clones sequenced, had several runs of a seven-nucleotide repeat identical to the *A. thaliana* telomeric repeat (5′TTTAGGG3′). This is noteworthy since in tomato the consensus telomere repeat sequence was determined to be TT(T/A)AGGG (Ganal et al. 1991). The tomato TR sequences were obtained by genomic sequencing of a DNA fraction enriched for telomeres, therefore it is possible that subtelomeric repeats, very similar to TRs, contributed to the sequence. Modified telomeric repeats at the borders between TRs and TASs have been reported for *Chlamydomonas reinhardtii* (Petracek et al. 1990) and human chromosomes (de Lange et al. 1990; Weber et al. 1990). Among the barley subtelomeric clones sequenced, four out of 18 repeats were imperfect (4 or 2 Gs instead of 3). This suggests that modified TRs are a common feature of subtelomeric sequences in eukaryotic chromosomes.

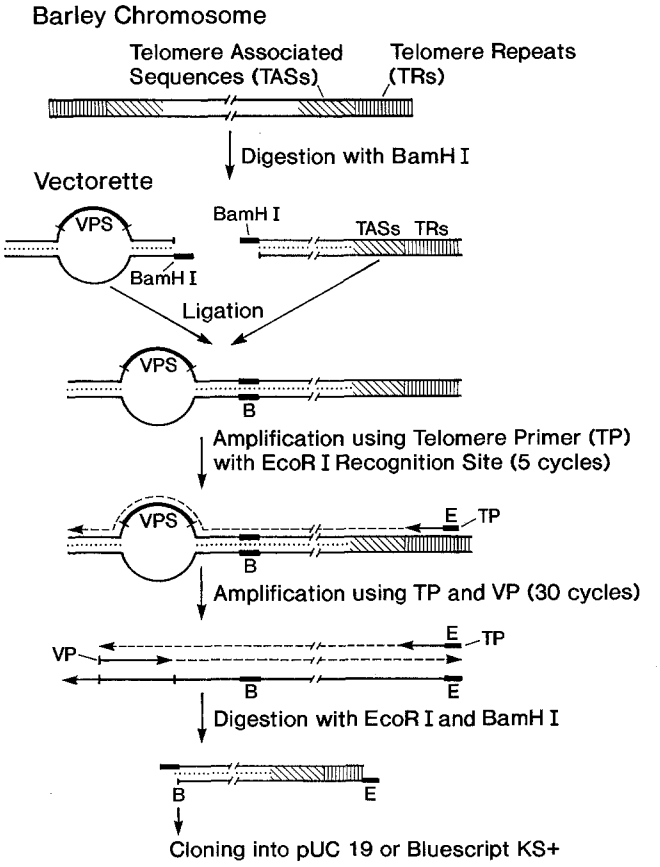

Fig. 1. Strategy for cloning barley telomere-associated sequences. Barley cv. Steptoe DNA was digested with *Bam*HI and ligated to a Vectorette designed as a partially double-stranded oligonucleotide adapter carrying a *Bam*HI compatible end and a single stranded region identical to the Vectorette Primer Sequence (VPS). This design assures that there is no sequence complementary to the Vectorette Primer (VP) until the strand complementary to the VPS sequence has been synthesized from the Telomere Primer (TP)

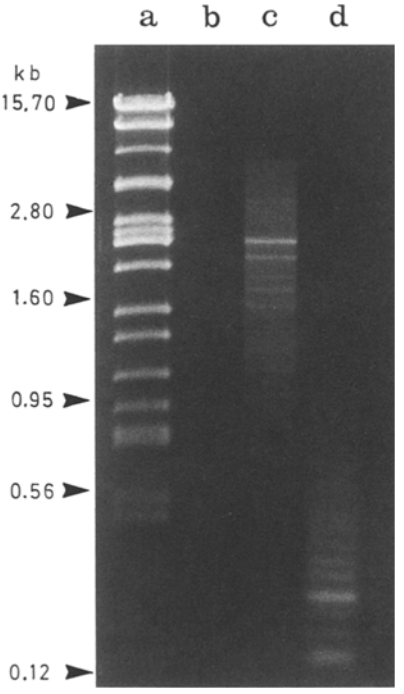

Fig. 2. Comparison of products obtained with barley DNA ligated to the Vectorette as template and amplified with telomere primer (TP; lane b), vectorette primer (VP; lane c) and TP + VP (lane d). Lane a – molecular weight markers

The barley subtelomeric clones described had only a few TRs and the 5′ -3′ orientation of the repeats was the same as determined for other eukaryotic chromosomes. The small number of TRs present in these clones indicates that our method is specifically directed towards sequences adjacent to the telomeric repeats. Although annealing of the telomere primer could occur anywhere along the TRs, the primer closest to the TR/TAS junction would presumably be favored for initiation of synthesis. Clones isolated from human chromosomes by a different method had long stretches of TRs both in proper and inverted polarity (Weber et al. 1990).

Clones pAkp2 and pAkp6 were unique, while clones pAkp3, pAkp17 and pAkp18 showed a high degree of homology among themselves and to three members of the HvRT family of repetitive sequences isolated from barley (Belostotsky and Ananiev 1990) (Table 1). HvRT sequences are organized into 118 bp tandem repeats present in 7×10^5 copies in the barley genome (Belostotsky and Ananiev 1990). Alignment of clones pAkp3 and pAkp18 to the consensus sequence for HvRT family showed that the sequence homology ends about 30 nucleotides before the TRs start. However, in clone pAkp17 TRs interrupt the consensus sequence of the HvRT repeat. Close physical proximity of tandemly arranged repetitive sequences and TRs seems to be another characteristic of subtelomeric regions shared by barley and other eukaryotes including yeast (Chan and Tye 1983) and *Plasmodium* (Corcoran et al. 1988).

The hybridization pattern obtained with the HvRT-like clones suggests that these sequences are arranged in tandem arrays and form higher order structures with other sequences (Fig. 3). This suggestion is supported by the observation that clone pAkp6 produced a similar hybridization pattern although it has no sequence homology with the HvRT family. The hybridization intensity obtained with pAkp6 was much lower than that with the HvRT-like clones, suggesting that this clone represents another, less abundant, class of repetitive sequences which are interspersed with members of the HvRT family. The HvRT clones and pAkp6 detected a high level of polymorphism over a range of barley cultivars and restriction enzymes. Hypervariability of TASs has been reported for numerous species including yeast, tomato and humans and a high rate of recombination of these sequences was postulated to account for the high level of variability (Zakian 1989; Broun et al. 1992).

Table 1. Nucleotide sequence comparison between barley subtelomeric clones and 3 members of the HvRT family of barley repetitive sequences

	pAkp3 %	pAkp17 %	pAkp18 %
HvT01	85.2 (149)	94.6 (149)	75.2 (129)
HvT02	88.1 (177)	80.9 (173)	66.0 (100)
HvT06	82.5 (120)	79.0 (119)	74.8 (107)

Sequence identity for each pair-wise comparison is given in % and the length of the overlap compared in bp (in parentheses). The HvT clone sequences are from Belostotsky and Ananiev (1990)

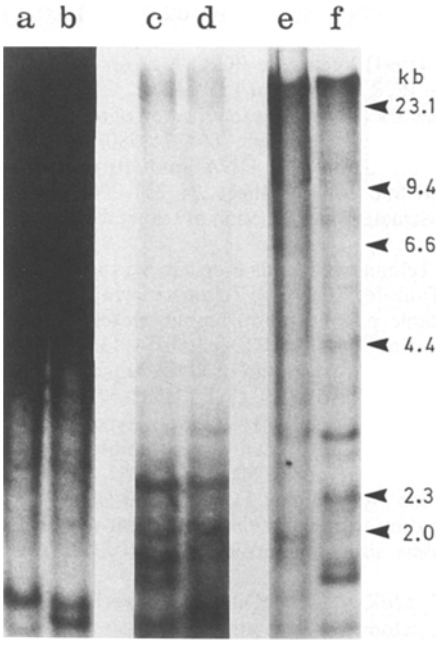

Fig. 3. Polymorphisms detected by barley subtelomeric clones and used for mapping. Genomic DNA from barley cv. Steptoe (lanes a, c, and e) and cv. Morex (lanes b, d, and f) was digested with *Xba*I (lanes a and b), *Taq*I (lanes c and d) and *Eco*RI (lanes e and f). The filters were hybridized with pAkp3 (lanes a–d) and pAkp6 (lanes e and f). Films were exposed with intensifying screens at −70° C for 24 h (lanes a and b), 7 h (lanes c and d) and 48 h (lanes e and f)

Polymorphisms detected by clones pAkp3 and pAkp6 behaved like Mendelian factors segregating in the expected 1:1 ratio. The pAkp3 *Taq*I and *Xba*I polymorphisms mapped most distally on the plus (short) arms of chromosome 2 and 4, respectively, and an *Eco*RI polymorphism detected by pAkp6 was mapped most distally on the plus (β) arm of chromosome 1 (Kleinhofs and Kilian 1992, and unpublished results). These results indicate that standard RFLP techniques are suitable for mapping TASs. These markers, due to their technical simplicity, should be superior to pulsed field gel electrophoresis for mapping telomeres.

We conclude that the barley clones described represent telomere-associated sequences based on characteristics that they share with other TASs and because they map to the ends of three barley chromosomes. The strategy described represents a simple and effective means to isolate telomere-associated sequences that should find general application for many eukaryotes.

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