No Conserved Homoeologous Regions Found in the Sugar Beet Genome

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ABSTRACT

Whether conserved homoeologous regions exist in the sugarbeet (*Beta vulgaris* L.) genome was investigated by RFLP analysis. Clones from a sugarbeet *Pst* I library were hybridized to genomic DNA at standard and low stringency. The number of loci detected by each clone was determined by evaluation of RFLP band patterns in segregating F_2 populations. Two separate experiments were carried out: 1) A total of 505 clones that had given rise to simple band patterns on screening blots and been used as RFLP probes at standard stringency in a previously reported RFLP mapping project were re-evaluated for their DNA sequence copy number. The 43 clones for which an exact copy number could not be determined were re-analysed at standard stringency in a different F_2 population. In addition, the DNA sequence copy number for 50 clones that showed more complex band patterns on screening blots was determined by RFLP analysis at standard stringency. 2) The DNA sequence copy number of 136 selected clones, evenly distributed over the previously reported RFLP map, was determined at low stringency. The two experiments revealed one duplicated sequence at standard stringency and one additional duplicated sequence at low stringency. Furthermore, 475 of the clones were confirmed at standard stringency and 94 at low stringency as being single-copy sequences. The clones distributed randomly over the nine linkage groups of sugarbeet. The two duplicated sequences identified mapped to different regions and were surrounded by single-copy sequences. Thus, no major homoeologous regions in the sugarbeet genome were revealed.

Additional Key Words: *Beta vulgaris,* genome structure, homoeology, RFLP analysis

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Sugarbeet (*Beta vulgaris* L.) is a diploid species with a haploid chromosome number of nine. This is also the basic chromosome number of other *Beta* species, and of species in several closely related genera such as *Chenopodium* and *Atriplex*. The karyotype of sugarbeet consists of similarly sized metacentric or submetacentric chromosomes that generally lack pronounced landmarks (Bosemark and Bormotov 1971; Cistue et al. 1985). The small size of the chromosomes is reflected by a haploid genome size of 1.2×10^9 basepairs (Bennet and Smith 1976). The genome consists of approximately 60% repetitive DNA (Flavell et al., 1974), much of it organized as satellite DNA in tandem arrays of a few hundred bp (Schmidt et al. 1991; Schmidt and Heslop-Harrison 1993). In addition, the repetitive DNA contains di, tri-, and tetra-nucleotide microsatellite repeats (Schmidt et al. 1993; Mõrchen et al. 1996), and several types of interspersed retrotransposons (Schmidt et al. 1995).

Information on the structure and function of the sugarbeet genome has been generated in molecular marker studies aimed at constructing linkage maps of the genome (Pillen et al. 1993; Barzen et al. 1995; Uphoff and Wricke 1995; Halldén et al. 1996; Nilsson et al. 1997). In the most extensive map of sugarbeet, involving more than 400 RFLP markers covering 621 cM (Halldén et al. 1996), the markers formed one dense cluster for each linkage group, indicating recombination in sugarbeet tends to be localized towards the ends of the chromosomes. This pattern has also been confirmed using RAPD markers (Nilsson et al. 1997). Resistance genes and genes determining annual growth habit, monogerm seed, hypocotyl color and fertility restoration have been located on several of these maps (Pillen et al. 1993; Barzen et al. 1995; Uphoff and Wricke 1995; Halldén and Nilsson, unpublished results).

Compared with other marker technologies, RFLP analysis is well suited for comparative genome mapping because RFLP markers are codominant, phenotypically neutral, highly abundant, and highly reliable. In addition, since RFLP analysis is based on the detection of DNA polymorphisms through hybridization with cloned DNA sequences, diverged DNA sequences can be detected by use of a low stringency hybridization strategy. It is thus possible to map DNA sequences evolutionary related to each other, both within and between genomes. In the present study we utilize these advantages of RFLP analysis to study the genome structure of sugarbeet with respect to the

occurrence of conserved duplicated regions within the genome. We employ a previously reported sugarbeeet genomic library and RFLP map of sugarbeet (Halldén et al. 1996) as a basis for the study.

MATERIALS AND METHODS

Plant material. Eight sugarbeet lines, chosen to represent the typical variation in a sugarbeet breeding program, were screened for DNA polymorphisms. Five different lines were used to produce three segregating F_2 populations of 330, 222 and 133 individuals. The latter two populations formed the basis for the high density RFLP map of sugarbeet reported previously (Halldén et al. 1996).

Genomic library. The sugarbeet genomic library used in this study has been described in Hjerdin et al. (1994) and Halldén et al. (1996). It was produced by using the methylation sensitive restriction enzyme *Pst*t I to digest sugarbeet DNA and selecting DNA fragments in the range of 500-2,000 bp. The DNA fragments were ligated into pUC19 and were used to transform *E. coli.*

Clones of the genomic library had previously been hybridized to filters containing DNA from eight sugarbeet genotypes digested by the restriction enzymes *Eco* RI, *Hind* III and *Eco* RV. The genomic clones were grouped into three classes (Table 1), based on the complexity of the band patterns.

All clones not confirmed to be single-copy were checked for multiple inserts by restriction analysis, using *Pst* I. Recombinant plasmids were isolated according to the alkaline lysis method of Sambrook et al. (1989).

Table 1. Classification of library clones of sugarbeet (see Materials and Methods for a description of the library).

DNA sequence copy number	No. of clones	No. of clones evaluated in F_2 populations
Low	856	505
Medium	89	50
High	1154	—
Sum	2099	550

DNA isolation. Leaf tissue of parents and F_2 individuals was freeze-dried and ground in a coffee grinder to a fine powder. The powder was mixed with CTAB extraction buffer (0.7 M sodium chloride, 0.1 M Tris, pH 7.5, 0.01 M EDTA 1% (w/v) N-Cetyl-N-N-N-trimethylammonium bromide (CTAB) 1% (v/v) ß-mercaptoethanol) and incubated at 60° C for 60 min. Each sample was mixed with an equal volume of 24:1 chloroform to iso-amylalcohol and centrifuged for 10 min at 900 g. The DNA was precipitated using 0.8 volumes of isopropanol. The precipitated DNA was washed in 76% ethanol, 0.2 M sodium acetate for 20 min and rinsed in 76% ethanol, 0.01 M ammonium acetate. The DNA was dissolved in 0.01 M Tris, 0.001 M EDTA, pH 8.0 and centrifuged at 15,000 g for 10 min. The DNA concentration was determined flourometrically using the dye Hoechst 33258.

Restriction enzyme digestion and Southern transfer. The DNA samples were digested for 5 h using 5 u/µg DNA and the restriction endonucleases *Eco* RI, *Hind* III and *Eco* RV. The DNA fragments were separated by gel electrophoresis using 0.8% agarose gels (SeaKem ME), denatured for 30 min in 0.2 M sodium hydroxide, 0.6 M sodium chloride and neutralized for 30 min in 0.5 M Tris, pH 7.5 1.5 M sodium chloride. The DNA was transferred by Southern transfer to MSI-membranes using 0.025 M sodium phosphate as transfer buffer. The membranes were washed in 2 x SSC (SSC; 0.15 M sodium chloride, 0.015 M sodium citrate) and UV-crosslinked at 120,000 µJ.

Labelling and hybridization. PCR based labelling of DNA was performed using α-^{32}P-dCTP (3,000 Ci/mmol) (Güssow and Clackson 1989). Excess nucleotides were removed using Sephadex-G50 spin column chromatography. The probes were denatured by heating to 100° C for 10 min before being added to the hybridization buffer (10% (w/v) polyethyleneglycol (mol wt 8,000), 0.6 x SSC, 7% (w/v) SDS, 0.01 M sodium phosphate, 0.005 M EDTA 100 mg/ml denatured and sonicated salmon sperm DNA). Standard stringency hybridization was carried out overnight at 60° C. The membranes were washed three times at room temperature in 0.25 x SSC, 0.2% SDS and subsequently washed in the same buffer for 45 min at 60° C. Low stringency hybridization was carried out overnight in the same hybridization buffer as above at 50° C. The membranes were washed three times at room temperature in 0.5 x SSC, 0.2% SDS and subsequently washed in the same buffer for 45 min at 50° C. Hyper-film-MP film

was exposed to the membranes at -80° C. Probes were removed by washing the membranes in 0.1 M sodium hydroxide, 0.2% SDS for 20 min and then in 0.2 M Tris, pH 7.5, 0.2% SDS, 0.1 x SSC for 20 min.

Standard stringency experiment. All the autoradiograms produced during the construction of the previously reported RFLP map (Halldén et al. 1996) were re-evaluated to determine whether bands could be identified as alleles from a single locus or from two or more loci, or whether monomorphic bands made such a determination impossible. Clones for which the number of loci present could not be determined in the first two F_2 populations were re-analysed in a third F_2 population. The analysis was performed on DNA, obtained from the parents and 21 F_2 individuals, that was digested by the restriction enzymes *Eco* RI, *Hind* III and *Eco* RV. The investigation aimed at determining the DNA sequence copy number of the 505 clones analysed at standard stringency hybridization in the previous study, and at summarizing the map positions of confirmed single-copy and duplicated sequences in the map (Halldén et al. 1996). The clones of the *Pst* I genomic library used for constructing the RFLP map were exclusively of the low copy category, as determined in screening blots by their low band complexity (Table 1). To investigate whether additional duplicated sequences were present in the class that contained more complex band patterns, clones from the medium copy number class (Table 1) were selected. Since many of these clones gave rise to multiple bands, and hence, could be expected to contain medium copy DNA sequences, we selected 50 clones with the lowest band complexity. These were surveyed for three restriction-enzyme/F_2 population combinations. Each combination contained DNA from the two parents and the 21 F_2 individuals present in either of the two original mapping populations, digested with either *Eco* RI, *Hind* III or *Eco* RV. The combinations were selected in such a way that all the clones were assayed on both populations.

Low stringency experiment. A selection of 136 clones representing every third clone from the RFLP linkage map of Halldén et al. (1996) was surveyed at low stringency hybridization to identify divergent homoeologous regions of the genome. Three combinations of the restriction enzymes *Eco* RI, *Hind* III or *Eco* RV and of the two original F_2 populations were examined, as described for the previous experiment.

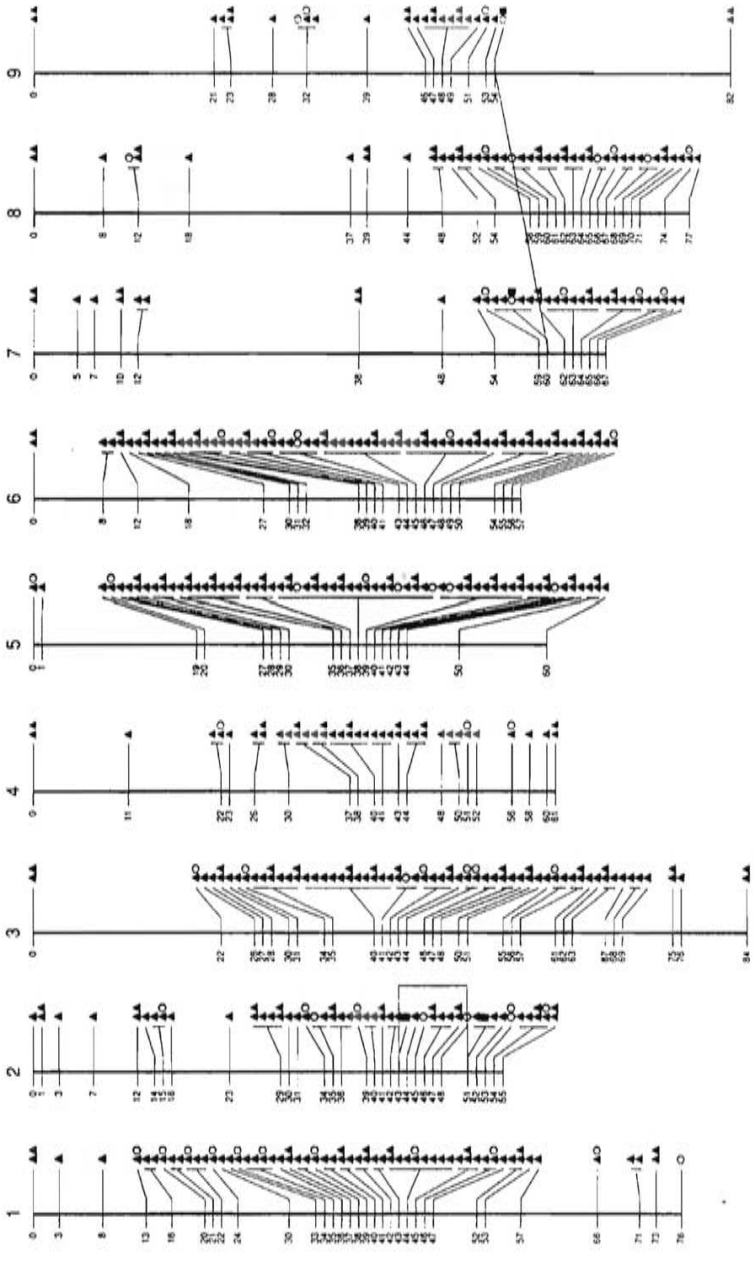
Mapping. The duplicated loci were mapped utilizing the JOINMAP program (Stam 1993), with a lod score of 3.0 being used for establishing linkage groups.

RESULTS

Standard stringency. As indicated, a previous study produced a high-density RFLP map of sugarbeet (Halldén et al. 1996). In the present study, autoradiographs used in the earlier mapping project were re-examined to identify clones for which all the bands could be identified as alleles from a single locus or from two or more loci. Only one duplicated sequence was found. In agreement with the results of the previous study, the duplicated sequence was SB12P-C02, represented by the two loci SB12P-C02 and 12C02B on linkage group 2 (Figure 1 and 2A). For 43 of the clones, not all the bands could be clearly dentified as being alleles from a single locus or from two or more lioci, often due to band patterns involving monomorphic bands. Such bands probably result from the presence of a restriction-enzyme recognition site in the DNA sequence of the probe, although they also may indicate the existence of other non-polymorphic locus. All the clones for which the number of loci could not be determined were re-analysed using a different F_2 population and three restriction enzymes. This analysis confirmed that 14 of these clones contained a single-copy DNA sequence. No additional duplicated loci were detected. In summary, 475 of the clones gave rise to band patterns for which all bands could be interpreted genetically as alleles from a single locus and only one clone revealed a duplicated sequence. The remaining 29 clones which originally gave rise to one or more additional monomorphic bands, also did so upon re-investigation. The distribution of these three categories among 411 mapped markers is shown in Figure 1. The mapped markers for which the DNA sequence

Figure 1 (Opposite). The distribution of RFLP markers when classified according to their DNA sequence copy number as revealed by RFLP analysis at standard (symbols in the left columns) and low stringency hybridization (symbols in the right columns). Symbols at standard and low stringency hybridization represent genomic clones confirmed as being either single-copy DNA sequences (▲) or duplicated sequences (■), as well as genomic clones for which the DNA sequence copy number could not be determined (○). The lines represent clones identifying two loci. The RFLP map is from Halldén et al. (1996). The map distances are based on the Kosambi (1944) function.

Figure 1.



copy number could not be determined did not appear to be clustered. In particular, very few of the markers were located in the vicinity of the two loci that represented the duplicated sequence.

The sugarbeet library used in the initial mapping effort contained a total of 856 clones categorized as containing low copy DNA sequences (Table 1). All the clones of this category that revealed polymorphisms in the two mapping populations were used for mapping. Since 94% of the clones gave rise to band patterns in which the number of loci could be determined and since only one clone containing a duplicated sequence was identified, we conclude that the low copy category contains very few duplicated sequences. To determine whether duplicated sequences were present at a higher frequency in the medium copy category, 50 clones from this category were exam-

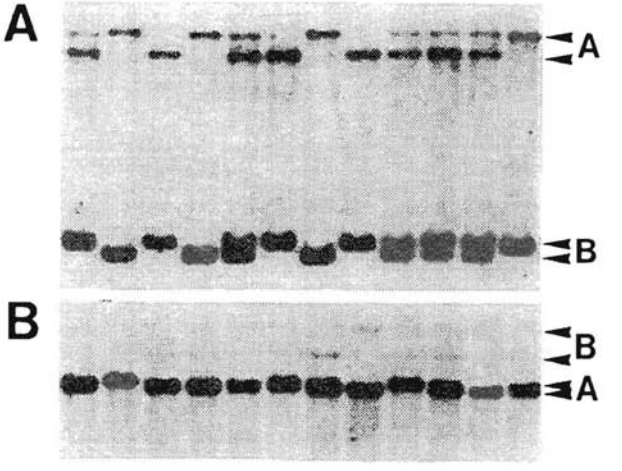


Figure 2 Examples of hybridization patterns of genomic clones representing duplicated DNA sequences. Arrowheads mark the alleles of the respective RFLP locus. A) Clone SB12P-CO2, revealing two RFLPs, A and B, of similar hybridization intensities at standard stringency hybridization. B) Clone SB37P-A01, revealing two RFLPs, A and B, at low stringency hybridization. The RFLP of locus B shows a less intense hybridization signal than the RFLP of locus A.

mined for the presence of duplicated sequences. The band patterns of the resulting autoradiographs showed 15 of the clones to contain single-copy DNA sequences, whereas 21 of the clones where shown to contain DNA sequences with a sequence copy number higher than two. The 14 remaining clones gave band patterns in which not all bands could be assigned to alleles. No clones containing a duplicated sequence were identified.

Low stringency. Approximately every third marker (in total 136 markers) were selected from the RFLP map of Halldén et al. (1996) (Figure 1). The corresponding clones were hybridized at low stringency to three restriction-enzyme/F_2 population combinations (see Materials and methods). The low stringency condition resulted in a pronounced background hybridization of 67% of the clones. The remaining clones displayed background patterns similar to those obtained under standard stringency conditions. Low stringency hybridization led to the detection of one additional, potentially duplicated DNA sequence, SB37P-A01, whereas 94 sequences gave single-copy hybridization patterns. The remaining 41 sequences gave band patterns in which not all the bands could be clearly identified as alleles from one locus only, e. g. due to the presence of monomorphic bands. The duplicated sequence detected at low stringency was not associated with the duplication detected at standard stringency but rather was represented by two loci mapping to linkage groups 7 and 9 (Figure 1). In contrast to the duplication detected at standard stringency, for which all the bands had the same intensity (Figure 2A), the duplication detected at low stringency showed one set of strong bands and one set of weak bands, corresponding to the alleles of the first and the second locus, respectively (Figure 2B).

DISCUSSION

The sugarbeet genome was investigated for the presence of conserved homoeologous regions by use of RFLP analysis at standard and low hybridization stringency. No such duplicated regions of the genome were detected when the RFLP band patterns in three segregating F_2 populations were examined. Only two duplicated DNA sequences were found and mapped. In contrast, 475 genomic clones at standard stringency and 94 clones at low stringency were confirmed single copy sequences. These were distributed evenly over the sugarbeet RFLP map. Thus, no ancient polyploidization events or major

duplications were revealed. This result is in agreement with that of Pillen et al. (1992), who observed that very few duplicated sequences were found in an RFLP map with 115 markers. In contrast, Barzen et al. (1992) reported that 34 genomic markers out of 92 mapped to more than one locus. All the RFLP fragments from the 48 individuals studied were mapped as dominant markers. Clearly this should lead to situations in which a codominant locus is mapped as two separate loci that are several centimorgans apart, particularly since the two alleles mapped independently of each other are in the repulsion phase (cf. Nilsson et al. 1997).

One duplicated sequence, SB 12P-C02, was identified by RFLP analysis at standard stringency. The corresponding loci mapped 8 cM apart on linkage group 2. Only minor differences in band intensity were detected for the alleles of the two loci, indicating a truly duplicated sequence. Most of the DNA sequences tightly linked to these loci were confirmed single-copy sequences. This indicates that the duplicated sequence was not associated with a large homoeologous region of the genome.

The very low frequency of duplicated sequences detected at standard stringency raises the question of bias against duplicated sequences in the selection of clones for mapping. Since all of the mapped markers were recruited from the low copy number class, one could argue that, nevertheless, there are a number of duplicated regions in the genome but that the corresponding DNA sequences are found in the medium copy number class that was not used for mapping. This enigma was addressed by evaluating the hybridization patterns of 50 medium number clones at standard stringency. The band patterns of the resulting autoradiographs showed 15 of the clones to contain single-copy DNA sequences, whereas 21 of the clones contained DNA sequences with a sequence copy number higher than two. Not all bands of the 14 remaining clones could be assigned to alleles. No clones containing a duplicated sequence were identified. Hence, the duplicated sequence mapped at standard stringency was not the result of a bias in the selection of genomic clones for mapping, but reflected the scarcity of duplicated sequences present in the sugarbeet genome.

A total of 136 clones, evenly distributed over the map, was investigated at low stringency. Hjerdin et al. (1994) showed 70% of the sugarbeet clones to hybridize at low stringency to genomic DNA

from the genus *Atriplex*. Ancient polyploidization or macrostructural duplication events would have been identified using this strategy. However, only one additional duplicated sequence was revealed. Since the hybridization intensity is weaker for the second locus, this locus may simply represent a similar DNA sequence that was cross-hybridized by chance. In addition, RFLP analysis confirmed that, at low stringency hybridization, 94 of the 136 clones investigated were single-copy. These confirmed single-copy clones were evenly distributed over the RFLP linkage map.

Polyploidy is a basic phenomenon in the evolution of genome size and structure. However, once a polyploid has been formed, diploidization through drift and selection begins. This process is illustrated by the occurrence of a series of species. On the one hand, there are species such as *Brassica napus, Triticum aestivum* and *Avena sativa* that can readily be identified by cytogenetic techniques as being relatively recent polyploids. On the other hand, the diploid species to which the ancient polyploids belong, such as *Zea mays,* and *Brassica nigra,* are only detected when molecular markers are used to study their genome structure (Whitkus et al. 1992; Truco and Quiros 1994). In *Beta vulgaris,* not even a low stringency hybridization reveals any polyploidization events. This observation is supported by the fact that in Chenopodiaceae the basic chromosome number of nine appears to be almost universal. This does not mean that translocations and inversions in the *Beta vulgaris* lineage may not have been fixed within the evolutionary pedigree of the Chenopodiaceae. This can only be determined by comparative mapping of species within the family. Our overall conclusion is that *Beta vulgaris* possesses a genuinely diploid genome that has been relatively undisturbed by major macrostructural rearrangements.

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