Theor Appl Genet (1989) 78:847–851

Use of restriction fragment length polymorphism to fingerprint beets at the genotype and species levels

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Received June 13, 1989; Accepted June 27, 1989
Communicated by H.F. Linskens

Summary. cDNA probes have been used to assess genetic variation in beet using hybridisation techniques that detect restriction fragment length polymorphism. Probes have been identified which differ in the levels of variation that they can detect (i) within closely related genetic material of sugar beet, and (ii) between sugar beet and a taxonomically distant *Beta* species.

Key words: Sugar beet – *Beta nana* – *Beta vulgaris* – RFLP

Introduction

The availability of useful genetic markers in sugar beet and related beets is low in comparison with many other crop plants (Nagamine and Ford-Lloyd 1989). Recent studies of isozyme variation in sugar beet have proved to be useful for following genetic recombination in interspecific hybrids (Oleo et al. 1986) involving sugar beet, as well as for monitoring the inheritance of resistance genes (Jung et al. 1986) and for studying patterns of genetic variation (Van Geyt and Smed 1984; Nagamine et al. 1989).

There is now growing evidence to suggest that DNA markers that are based on RFLPs uncover more differences between individual plants than do protein markers, and are also able to detect differences between species (Bernatsky and Tanksley 1989).

There is considerable divergence within results from different studies focussing on a range of crop plants, regarding the number of random DNA probes which need to be screened, together with the number of restriction enzymes required to detect variation in restriction fragments in closely related and more distantly related genetic material (Bernatsky and Tanksley 1989). We have examined restriction fragment length polymorphism in a small amount of sugar beet and wild beet species germplasm, which has been specifically selected to contrast in the degree of genetic relatedness, to determine the number of random cDNA probes which have to be screened in order to discriminate between genotypes and species. Such information is currently unavailable for sugar beet, and should provide impetus to the prospects of utilising RFLP markers for monitoring progress of selection within breeding programmes.

Materials and methods

Plant material

Two accessions of sugar beet – a tetraploid Polish breeding line (B117) and a triploid cultivar, Sharpe's Klein Mono (B78) – together with a wild *B. vulgaris* ssp. *maritima* accession (B95) from the UK and one accession of *B. nana* originating from Greece, were grown under glasshouse conditions for approximately 15–20 weeks. Seven to eight plants of each were grown for further analysis, except for the *B. nana*, where fewer plants were available because of the rarity of the seed and difficulties with germination.

DNA isolation, digestion and Southern transfers

DNA was isolated from leaf material using the procedure of Dellaporta et al. (1983). Purified DNA (approximately 10 µg) was digested with HindIII (NBL), according to the manufacturers' instructions, at 5 units per µg DNA for 90 min, followed by the addition of a further 5 units per µg for a further 90 min. DNA fragments were separated on a 0.7% agarose gel and transferred to Hybond-N hybridisation membrane (Amersham International) and fixed at 80 °C for 2 h according to the manufacturers' instructions.

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Construction of cDNA libraries

For extraction of RNA, the normal precautions were taken to avoid degradation by RNases. Twenty grams of the sugar beet cultivar Bush Mono G leaf material was frozen in liquid nitrogen and homogenised in a mortar before addition of 50 ml of homogenisation buffer (0.4 *M* NaCl, 50 m*M* TRIS-HCl, pH 9.0, 1% sodium dodecyl sulphate (SDS), 10 m*M* dithiothreitol, 5 m*M* Na_2 EDTA, 20 units ml^{-1} heparin, 1 m*M* aurintricarboxylic acid) and 25 ml of phenol saturated with homogenisation buffer and containing 10% m-cresol. The mixture was then homogenised to a paste in a tissue disintegrator and centrifuged at 10,000 × g for 10 min. The aqueous phase was extracted twice with 20 ml of phenol/chloroform, then made 2 *M* with respect to LiCl and incubated at 4 °C overnight. After centrifugation at 10,000 × g for 10 min, the pellet was washed twice with 5 ml 3 *M* sodium acetate, pH 5.2, before resuspension in H_2O. The RNA was then re-precipitated with LiCl and centrifuged down as before. The pellet was washed three times with 70% ethanol at room temperature, dried under vacuum and resuspended in water.

One gram of oligo (dT) cellulose (Sigma) was poured into a small chromatography column to give a 1-cm bed height, and the column was washed with water, 0.1 *M* NaOH containing 5 m*M* Na_2 EDTA, and again with water. When the effluent pH was below 8.0, the column was equilibrated with 1 × high LiCl buffer (20 m*M* TRIS-HCl, pH 7.6, 0.5 *M* LiCl, 1 m*M* Na_2 EDTA, 0.1% SDS). The RNA sample was then incubated at 65 °C for 5 min, an equal volume of 2 × high LiCl buffer added, then allowed to cool to room temperature before being applied to the column. The column was washed with 5–10 vol of 1 × high LiCl buffer, followed by 4 column vol of low LiCl buffer (as 1 × high LiCl buffer but containing only 0.1 *M* LiCl) to remove poly $(A)^-$ RNA. Poly $(A)^+$ mRNA was eluted with 2–3 column vol of elution buffer (0.05% SDS, 1 m*M* Na_2 EDTA, 10 m*M* TRIS-HCl, pH 7.6), and precipitated by the addition of 1/10 vol of 3 *M* sodium acetate, pH 5.2, and 2 vol of ethanol at –20 °C. The RNA was collected by centrifugation at 15,000 × g for 15 min, washed with 70% ethanol at room temperature, dried under vacuum, then taken up in water.

Using the poly$(A)^+$ RNA population as template, double-stranded cDNA was synthesised employing the RNase H method of Gubler and Hoffman (1983) using a cDNA synthesis kit (Pharmacia LKB Ltd.). Linkered cDNA was cloned into the EcoR1 site of Igt10 (Stratagene), packaged using Gigapack Plus extracts (Stratagene) and used to transfect *E. coli* strain C600 *HflA*- cells.

Preparation of probes

A random selection of recombinant λgt 10 clones were grown up in liquid medium and DNA was extracted using the mini-preparation protocol of Grossberger (1987). Phage DNA was digested using EcoR1 and the DNA fragments were separated using a low melting point agarose (Sigma) gel. cDNA inserts were excised from the gels and labelled using ^{32}P-dCTP (Amersham International) in the random hexanucleotide primer-directed synthesis method of Feinberg and Vogelstein (1983, 1984).

Hybridisation technique

Filters were pre-hybridised in 5 × SSC, 1 × Denhardt's solution, 40% formamide and 100 μg/ml sheared salmon sperm DNA (Maniatis et al. 1982) at 42 °C for 4 h. Denatured, labelled cDNA probe was added directly to the pre-hybridisation mix and the incubation was continued at 42 °C for a further 16 h. Filters were initially washed twice in 2 × SSC, 0.5% SDS at room temperature for 30 min. Filters were then washed twice in 0.5 × SSC, 0.5% SDS at 50 °C for 30 min. Damp filters were covered with Saran wrap (Dupont) and used to expose Hyperfilm-MP X-ray film (Amersham International) in cassettes fitted with intensifying screens at –70 ° for appropriate periods. Following development of film, filters were stripped for reprobing according to the manufacturers' instructions.

Results

Probe L11/2/5, approximately 400 bp in length, hybridised with two DNA fragments, the mobilities of which were invariant in all the material tested (Fig. 1). Probe L39S, approximately 650 bp in length, hybridised strongly to bands corresponding to 1.0 and 3.0 kb, and weakly to a third band of approximately 2.2 kb in the Sharpe's Klein Mono (B78) and Polish Breeding line (B117) material (Fig. 2). No variation was observed within these samples using this probe. However, the same probe revealed polymorphism within the ssp. *maritima* accession. Whilst the 1.0-kb band appeared constant, the sizes of the fragments at approximately 3.0 kb varied among some of the individuals tested. The weakly hybridising 2.2-kb third band evident in B78 and Polish Breeding line samples was just visible in some tracks. Probe L39 hybridised to two bands in the *B. nana* samples, one of which was equivalent to the 1.0-kb band common to the other beet samples tested whilst the other, with a mobility corresponding to approximately 6.0 kb, was not observed in the other tracks.

Probe L24, complementary to a highly abundant leaf mRNA, hybridised with multiple bands from both B78 and the Polish Breeding line material (Fig. 3), suggesting that this cDNA represented a mRNA species from a multigene family. Obvious variation in the hybridisation patterns for B78 samples was observed, with four different profiles being apparent (lanes C-F). In contrast, the hybridisation pattern from the Polish Breeding line material was constant.

Polymorphism was also detected within the spp. *maritima* samples using probe L24; the extent is not clear because of the poor signals from some of the tracks. Once again, the *B. nana* samples clearly differed from the other material tested. Although signs of a multigene family were evident, the bulk of the hybridisation was concentrated in an area which has the appearance of a doublet at approximately 4.0 kb.

Discussion

Three random cDNA probes have been isolated which contrast markedly in their capabilities of detecting restriction fragment length polymorphism within sugar beet and wild *Beta* species. One probe (L11/2/5) clearly

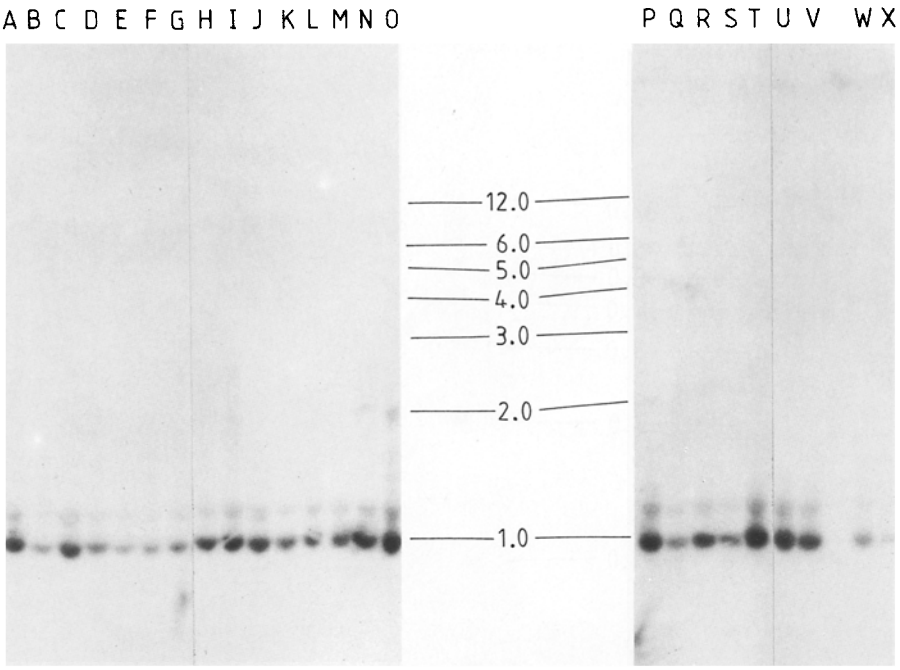

Fig 1. RFLP patterns resulting from the hybridisation of cDNA clone L11/2/5 to *Hind*III-digested total genomic DNA from plants originating from two sugar beet, one *B. vulgaris* ssp. *maritima* and one *B. nana* accessions. *Lanes A–G*, sugar beet Sharpe's Klein Mono (B78), plants 1–7, respectively; *lanes H–O*, sugar beet Polish breeding line (B117), plants 1–8, respectively; *lanes P–V*, *B. vulgaris* ssp. *maritima* (B95), plants 1–7, respectively; *lanes W and X*, *B. nana*, plants 1 and 2. Molecular weight markers are in kb

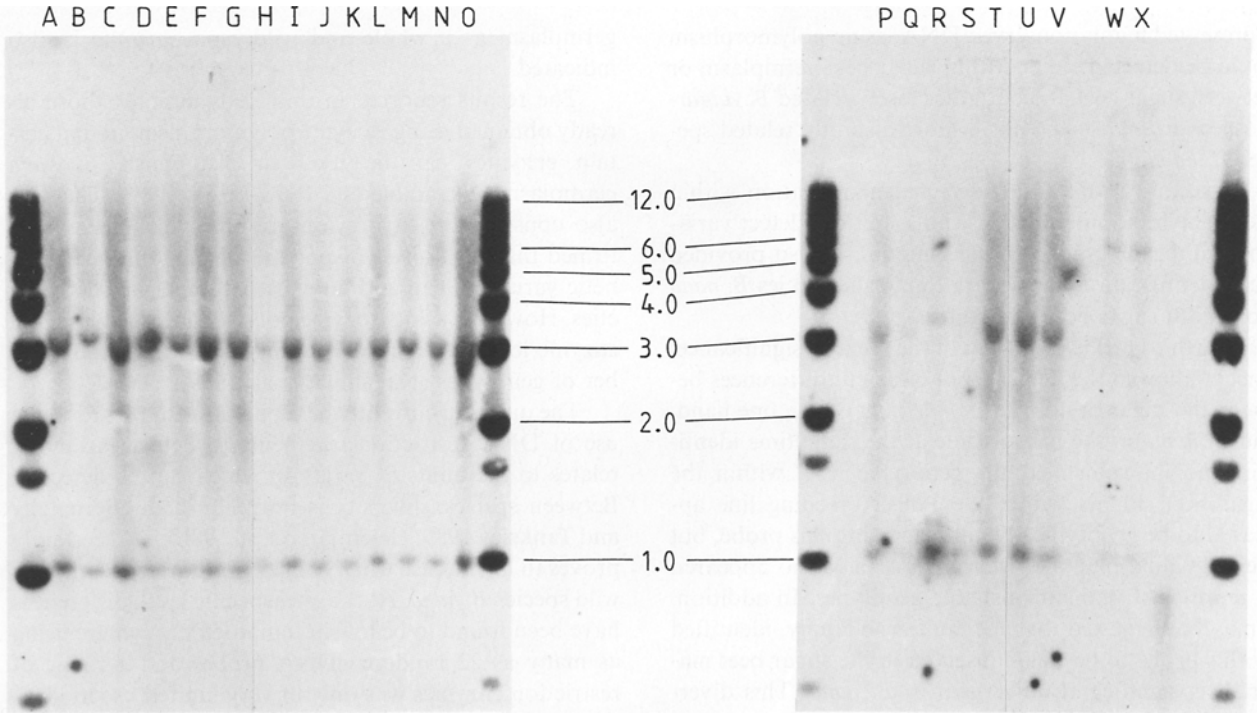

Fig 2. RFLP patterns resulting from the hybridization of cDNA clone L39S to *Hind*III-digested total genomic DNA from plants originating from two sugar beet, one *B. vulgaris* ssp. *maritima* and one *B. nana* accessions. *Lanes A–G*, sugar beet Sharpe's Klein Mono (B78), plants 1–7, respectively; *lanes H–O*, sugar beet Polish breeding line (B117), plants 1–8, respectively; *lanes P–V*, *B. vulgaris* ssp. *maritima* (B95), plants 1–7, respectively; *lanes W and X*, *B. nana*, plants 1 and 2. Molecular weight markers are in kb

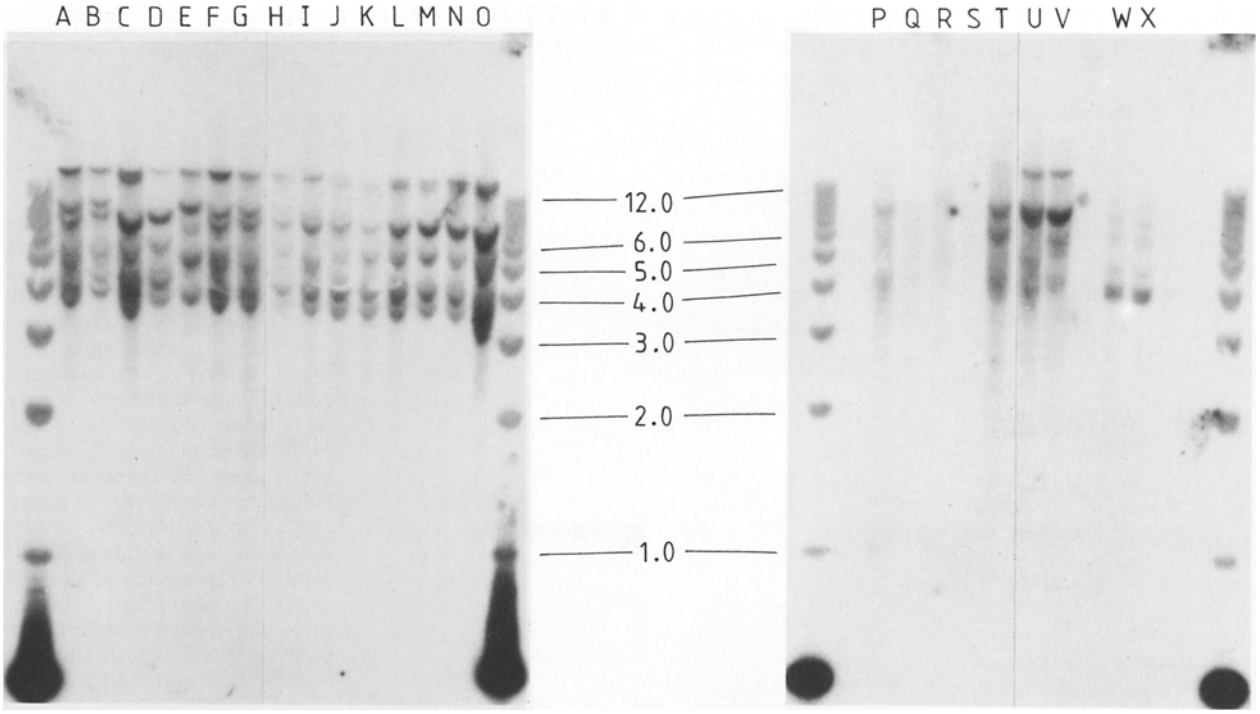

Fig 3. RFLP patterns resulting from the hybridization of cDNA clone L24 to *Hind*III-digested total genomic DNA from plants originating from two sugar beet, one *B. vulgaris* ssp. *maritima* and one *B. nana* accessions. *Lanes A–G,* sugar beet Sharpe's Klein Mono (B78), plants 1–7, respectively; *lanes H–O,* sugar beet Polish breeding line (B117), plants 1–8, respectively; *lanes P–V, B. vulgaris* ssp. *maritima* (B95), plants 1–7, respectively; *lanes W and X, B. nana,* plants 1 and 2. Molecular weight markers are in kb

represented highly conserved DNA, as no polymorphism could be detected either within sugar beet germplasm or between sugar beet and both the closely related *B. vulgaris* ssp. *maritima* or the much more distantly related species *B. nana.*

A second probe (L39) detected no variation within the sugar beet material as a whole, but did detect variation within the ssp. *maritima* material. It also provided clear identification and separation of the species *B. nana* from both sugar beet and ssp. *maritima.*

The third probe (L24) was of the greatest significance, since it allowed the detection of clear-cut differences between the sugar beet and ssp. *maritima* on the one hand, and *B. nana* on the other, while at the same time identifying polymorphism at the genotypic level within the sugar beet. Plants within the Polish breeding line appeared to be genotypically uniform using this probe, but the individual plants of Sharpe's Klein Mono appeared to represent five distinguishable genotypes. In addition, it may be suggested that the multigene family, identified by this probe to be well-conserved in the sugar beet material, is significantly divergent in *B. nana.* This divergence was not only apparent with regard to copy number and HindIII sites, but also to reduced sequence homology, since this probe bound relatively more poorly to *B. nana* DNA. A close relationship between the sugar beet germplasm as a whole and wild ssp. *maritima* is also indicated.

The results reported in this study support those already obtained using isozyme polymorphism, in that certain enzymes can be used to distinguish *B. nana* germplasm (Nagamine and Ford-Lloyd 1989). They are also consistent with previous results which have confirmed that high levels of polymorphism, and hence genetic variation, can and do exist within sugar beet varieties. However, using isozyme polymorphism, at least ten enzyme loci were required to fingerprint the same number of genotypes (Nagamine et al. 1989).

The question currently being posed with regard to the use of DNA restriction fragments to detect variability relates to the limits of variation which can be detected. Between species, diversity is normally high (Bernatzky and Tanksley 1989; Helentjaris et al. 1985). This certainly proves to be the case when comparing *B. vulgaris* and the wild species *B. nana.* At the intraspecific level, differences have been found to be low in tomatoes, e.g., where using as many as 22 random cDNA probes and a range of restriction enzymes was only of very limited use in identifying genetic differences between inbred varieties (Helentjaris et al. 1985). In contrast, comparisons made using random cDNA probes in maize have shown polymorphism to be high, as 9 out of 16 probes have detected

polymorphism among inbreds using a single restriction enzyme (Burr et al. 1983).

It has been considered that the situation in maize may be unusual amongst crop plants, and that many crop plants may fall between tomato and maize with regard to the ease with which intraspecific RFLP can be detected (Bernatzky and Tanksley 1989). However, it seems clear from our results that sugar beet may well be comparable to maize in this respect. In this case we have detected significant variation at the genotype level and the species level, using one probe out of three tested and using only one restriction enzyme.

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