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Abundance and polymorphism of simple repetitive DNA sequences in *Brassica napus* L.

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Summary. The *Brassica napus* genome has been investigated by DNA fingerprinting with six synthetic oligonucleotide probes complementary to simple repetitive sequences, namely $(GATA)_4$, $(GACA)_4$, $(GGAT)_4$, $(CA)_8$, $(CT)_8$ and $(GTG)_5$. While all sequence motifs were found to be present in the *B. napus* genome, their organization and abundance varied considerably. Among the investigated probes, $(GATA)_4$ revealed the highest level of intraspecific polymorphism and distinguishes not only between cultivars but even between different individuals belonging to the same cultivar. In contrast, $(GTG)_5$, $(GACA)_4$ and $(GGAT)_4$ produced relatively homogeneous fingerprint patterns throughout different cultivars, while hybridization to $(CT)_8$ and $(CA)_8$ resulted in only a few weak bands superimposed on a smear. The isoschizomeric pair *Hpa*II and *Msp*I revealed the presence of methylated cytosines in the vicinity of $(GATA)_m$ repeats. The applicability of simple repetitive sequence polymorphisms as molecular markers for *Brassica* species is discussed.

Key words: *Brassica napus* – DNA fingerprinting – Simple repetitive sequences – Cultivar identification – DNA methylation

Introduction

The economic importance of crop species belonging to the genus *Brassica* has prompted the development of molecular markers that make it possible to differentiate between genotypes. Several molecular marker systems are now available for *Brassica* species, including isozyme (Arus et al. 1985; Mündges et al. 1990) and seed protein patterns (Gupta and Röbbelen 1986), nuclear RFLPs (Song et al. 1990, 1991; Landry et al. 1991), and RAPDs (Hu and Quiros 1991; Quiros et al. 1991). Several classes of tandemly repeated satellite and minisatellite DNA have also become attractive as molecular markers, but have not yet been exploited for breeding purposes in *Brassica* species. Whereas satellite DNAs can serve as valuable genome-specific markers, as demonstrated with the chromosome addition lines of beet (Schmidt et al. 1990) and somatic hybrids between potato and tomato (Schweizer et al. 1988), polymorphic minisatellites and simple sequence motifs, which are ubiquitous components of most if not all eukaryotic genomes (Epplen et al. 1991), represent useful target sequences for DNA fingerprint analyses (reviewed by Nybom 1991; Weising et al. 1991b).

Since the early studies performed by Beridze (1975), satellite DNAs have been thoroughly investigated in the Brassicaceae. For example, one particular satellite exhibiting a basic motif of about 175 bp in length has been detected in a variety of the members of this family (Grellet et al. 1986; Halldén et al. 1987; Reddy et al. 1989; Harbinder and Lakshmikumaran 1990; Lakshmikumaran and Ranade 1990; Sibson et al. 1991). Another, extremely AT-rich satellite has proven characteristic of the genomes of *B. nigra* and *Sinapis arvensis* (Gupta et al. 1990), and a species-specific satellite has recently been found in *B. campestris* (Iwabuchi et al. 1991).

In contrast to the wealth of data available on the organization of satellite DNAs, little is known about the occurrence and distribution of minisatellites and

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simple repetitive sequences in the Brassicaceae. To fill part of this gap, we have examined the organization of simple repetitive sequences in several species of the Brassicaceae by DNA fingerprinting with synthetic oligonucleotide probes. We showed previously that $(GATA)_m$- and $(GACA)_n$-repeats occur in the genome of *Brassica napus* (Weising et al. 1991a). Here we report on the abundance and polymorphism of six different simple repetitive sequence motifs in the genomes of different cultivars, individuals, and tissues, as well as in somaclonal regenerants, of oilseed rape (*Brassica napus* var. *oleifera*).

Materials and methods

Plant materials

Eight commercially available cultivars of rapeseed (*B. napus* L. var. *oleifera*) were analyzed: Ceres and Line (both supplied by Danish Plant Breeding, Denmark), Tower and Jet neuf (from the collection of the Danish Institute of Plant and Soil Science), Rally (Kleinwanzlebener Saatgut AG, FRG), Diplom, Hanna and Topas (supplied by Svalöf AB, Sweden). Plants were grown under greenhouse conditions. For some experiments, plants were regenerated from protoplasts as described by Poulsen and Nielsen (1989). For DNA isolation, either fresh or lyophilized plant material was used.

DNA isolation and purification

The DNA isolation procedure described by Saghai-Maroof et al. (1984) was modified as follows. Lyophilized or fresh plant material was ground in liquid nitrogen with a mortar and a pestle and dispersed in isolation buffer (2% w/v cetyltrimethyl ammonium bromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 0.2% v/v 2-mercaptoethanol) prewarmed to 60 °C. After 30 min incubation at 60 °C, the suspension was extracted once with chloroform: isoamyl alcohol (24:1) and centrifuged for 10 min at 5,000 g at room temperature. The aqueous phase was transferred to a new tube and nucleic acids were precipitated by adding 0.6 volumes of cold isopropanol. Precipitates were collected by centrifugation (30 min, 5,000 g, 4 °C), washed once with 76% ethanol, 10 mM ammonium acetate, and centrifuged as described above. Pellets were then dried, redissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), transferred to ultracentrifuge tubes and mixed with two volumes of gradient buffer (6.6 M CsCl, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 150 µg/ml ethidium bromide). CsCl density gradients were run at 45,000 rpm and 16 °C for 24 h in a Beckman VTi 65-2 rotor. DNA bands were removed with a syringe attached to a wide-gauge needle, and ethidium bromide extraction was performed within the syringe (Hofmann and Weising 1990). The final aqueous phase was diluted with two volumes of TE to avoid co-precipitation of CsCl, and DNA was precipitated with two volumes of ethanol. After 2 h at −20 °C, pellets were collected by centrifugation (30 min, 5,000 g, 4 °C), washed in 70% ethanol, dried and dissolved in an appropriate volume of TE buffer.

Oligonucleotide fingerprinting

Five micrograms of DNA from each sample were digested with *Taq*I, *Hin*fI, *Eco*RI, *Msp*I or *Hpa*II according to the supplier's instructions. Digested samples were loaded onto 1% agarose gels in TAE buffer (40 mM Tris-HCl, 20 mM sodium acetate, 1 mM EDTA, pH 7.8) and run at 4 °C and 1–2 V/cm for 20–24 h. After staining with ethidium bromide and photographing, the gels were denatured, neutralized, dried on a vacuum gel dryer, and hybridized to ^{32}P-endlabelled $(GATA)_4$, $(GACA)_4$, $(GGAT)_4$, $(CA)_8$, $(CT)_8$ and $(GTG)_5$ probes essentially as described (Ali et al. 1986; Schäfer et al. 1988). Melting temperatures (T_m) were calculated according to Thein and Wallace (1986). Hybridization and stringent washes were performed at $T_m - 5$ °C. After washing, gels were blotted dry, wrapped in Saran foil, and exposed to X-ray films (Kodak X-omat S or XAR) at −80 °C using intensifying screens. Before hybridizing to a new probe, gels were regenerated by washing in 5 mM EDTA at 60 °C (2 × 15 min).

Results

Abundance and polymorphism of simple repetitive motifs in the B. napus genome

To investigate the presence and polymorphic behaviour of different simple sequence motifs in the *B. napus* genome, *Hin*fI-digested DNA of three different rapeseed cultivars was consecutively in-gel-hybridized to

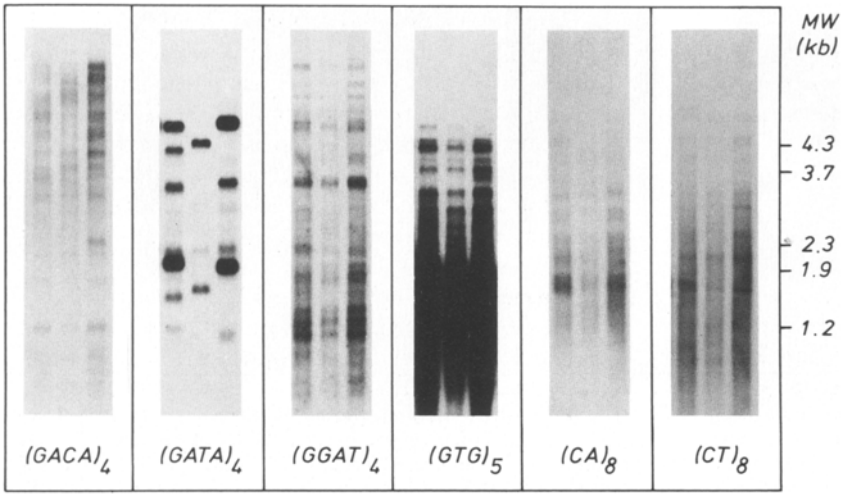

Fig. 1. Screening for the presence of simple sequence repeats in the genomes of *B. napus* var. *oleifera*, cv Hanna, cv Line and cv Rally (from left to right in each panel). Purified DNA was digested with *Taq*I, electrophoresed (5 µg per lane) and in-gel-hybridized to the indicated oligonucleotide probes. The same gel was used for all experiments. For rehybridization, former probes were removed by treatment with 5 mM EDTA. The sizes of molecular weight markers are given in kb

six different probes. The results showed that all tested motifs occur in *B. napus*, though at different abundances (Fig. 1). According to the intensities of hybridization signals, $(GTG)_n$-repeats are most abundant, followed by $(GATA)_m$-repeats. $(GACA)_4$, $(GGAT)_4$, $(CT)_8$ and $(CA)_8$ produced relatively weak signals that were only visible after several days of exposure using intensifying screens. Among the probes tested, $(GATA)_4$, was the most informative in two respects: (1) clear-cut banding patterns lacking any background smear were produced, and (2) polymorphic banding patterns made it possible to differentiate between cultivars. As compared to $(GATA)_4$, the hybridization patterns produced by all the other probes [$(GTG)_5$, $(GACA)_4$, $(GGAT)_4$, $(CT)_8$, and $(CA)_8$] were of inferior quality: signals were less distinct, and bands were usually superimposed on a smear. Moreover, with the exception of $(GACA)_4$, banding patterns were rather monomorphic throughout the different cultivars. In view of these results, $(GATA)_4$ and $(GTG)_5$, (producing the most variable and the strongest patterns, respectively) were selected for a more detailed analysis.

In order to optimize DNA fingerprint patterns, five different restriction enzymes were tested: *Taq*I, *Hin*fI; *Eco*RI, *Msp*I and *Hpa*II. The latter two enzymes are isoschizomers with different sensitivities to recognition site methylation (Kessler and Hoeltke 1986). Polymorphic $(GATA)_4$-patterns were produced by all enzymes (data not shown). *Eco*RI-generated fragments were clustered in the high molecular weight range and did not allow easy evaluation. Predominantly large fragments were also produced by the methyl-sensitive 'four-cutters' *Msp*I and *Hpa*II, suggesting a rare occurrence and/or a high level of cytosine methylation of CCGG motifs in the vicinity of the $(GATA)_m$ repeats. *Taq*I and *Hin*fI were found most suitable for oligonucleotide fingerprinting of *B. napus* DNA, since the $(GATA)_4$-positive fragments generated by these two enzymes were in a size range (i.e., between 0.5 and 5 kb) easily analyzable for length polymorphisms. Except for the methylation experiment (see Fig. 4), *Taq*I was routinely used for all subsequent experiments.

Inter- and intra-cultivar variability of $(GATA)_m$- and $(GTG)_n$-repeats

To investigate the potential of oligonucleotide fingerprinting for variety identification, eight cultivars (three individuals each) were compared by hybridization to $(GATA)_4$ and $(GTG)_5$ (Fig. 2). While almost no variation was found with $(GTG)_5$, the $(GATA)_4$ pattern proved to be very informative by distinguishing

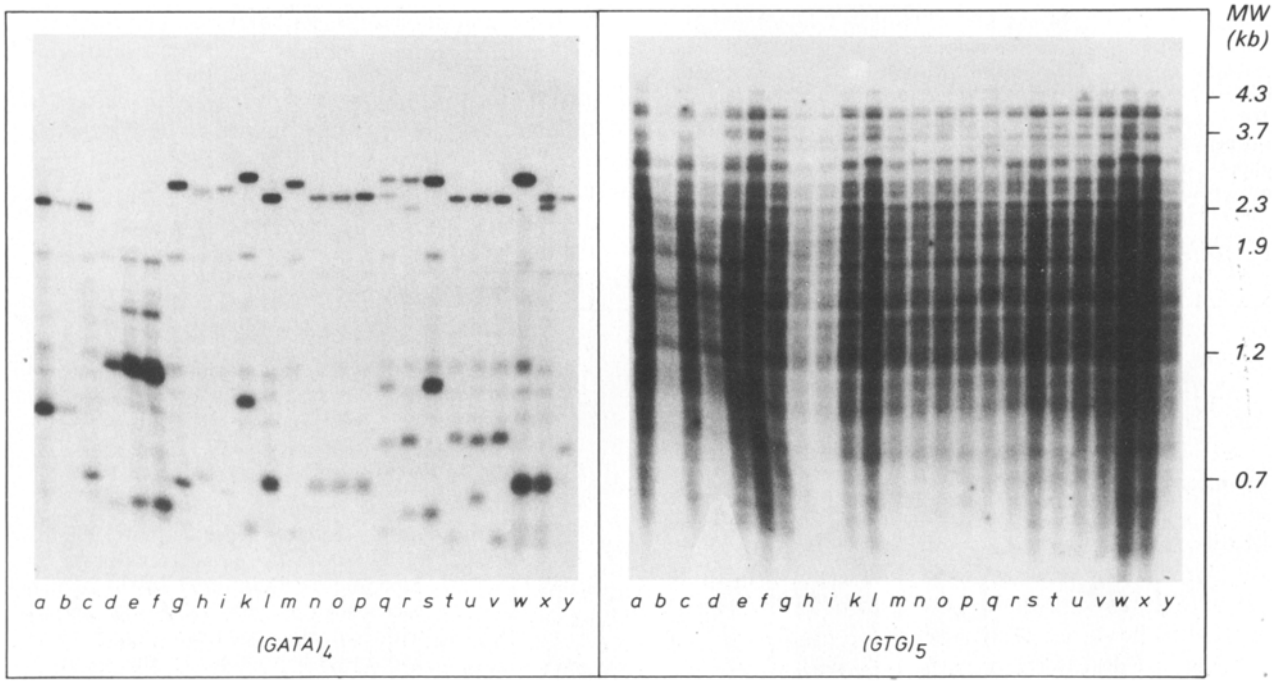

Fig. 2. $(GATA)_4$- and $(GTG)_5$-fingerprints of eight varieties of *B. napus* var. *oleifera* (three individual plants each): Ceres (*a–c*), Jet neuf (*d–f*), Tower (*g–i*), Rally (*k–m*), Diplom (*n–p*), Hanna (*q–s*), Line (*t–v*) and Topas (*w–y*). Purified DNA of each sample was digested with *Taq*I, electrophoresed (3 µg per lane), and in-gel-hybridized to the indicated probes. The same gel was used for both experiments. The sizes of molecular weight markers are given in kb

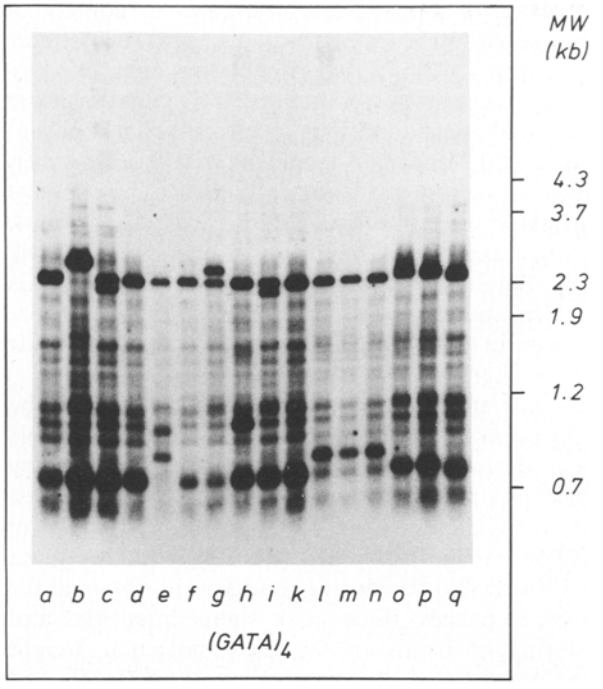

Fig. 3. $(GATA)_4$-fingerprints of individual plants of *B. napus* var. *oleifera* cv Topas (*a–l*), of different tissues derived from the same plant (*l*, leaf; *m*, stem; *n*, root), and of leaves from three individual plants regenerated from tissue culture (*o*, R19-1; *p*, R19-2; *q*, R19–372). Purified DNA of each sample was digested with *Taq*I, electrophoresed (5 µg per lane), and in-gel-hybridized to $(GATA)_4$. The sizes of molecular weight markers are given in kb

all cultivars from each other. However, except for Jet neuf (lanes d–f) and Diplom (lanes n–p), between-individual variability within cultivars was also observed. Thus, the applicability of $(GATA)_4$-fingerprints as 'identity cards' for cultivar identification is probably limited. To study the extent of intravarietal $(GATA)_m$-polymorphism in more detail, we examined the $(GATA)_4$-fingerprints of 11 individual plants derived from the variety 'Topas' (Fig. 3, lanes a–l). While the overall pattern 'architecture' appeared conserved, there are notable differences between individuals, and only individuals 'a', 'd', and 'k' actually looked identical. An interesting situation was encountered with the major bands around 2.3 kb. Here, either a single or a double band is present at slightly variable positions, suggesting different allelic states (homo- and heterozygosity) of a polymorphic locus with at least three alleles. A locus-specific probe will have to be developed to test this hypothesis.

Taken together, the results obtained with the $(GATA)_m$ repeats show that considerable variation still exists within cultivars of *B. napus*, as might be expected from the outcrossing properties of this species. Though being able to distinguish between cultivars, $(GATA)_4$ thus seems not suitable for cultivar identification. No obvious intracultivar variation was observed with $(GTG)_5$ (Fig. 2), $(GACA)_4$, and $(GGAT)_4$ (data not shown).

Somatic stability, somaclonal variation and DNA methylation

Another series of experiments was dedicated to the mitotic stability of $(GATA)_m$-fingerprints, and to the evaluation of methylated CCGG sites in the vicinity of $(GATA)_m$ repeats. To this end, DNA from leaves, stems and roots derived from the same individual (cultivar Topas) was cut with *Taq*I, *Msp*I and *Hpa*II, respectively. While *Hpa*II is sensitive to methylation at both cytosines of its target site CCGG, *Msp*I does not cut if the external C is methylated (Kessler and Hoeltke 1986). Hybridization of *Taq*I-digested DNA to $(GATA)_4$ resulted in uniform patterns for all three organs (Fig. 3, lanes l–n), thus demonstrating that $(GATA)_4$ fingerprints are somatically stable. The

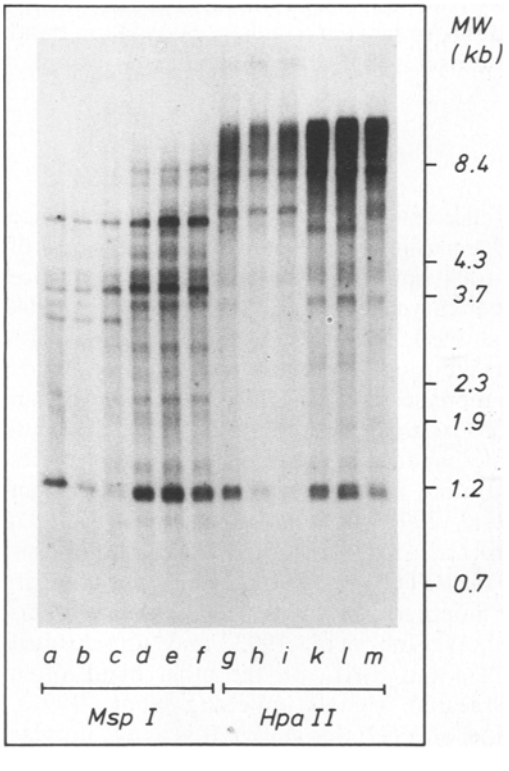

Fig. 4. Methylation state of CCGG sites in the neighbourhood of $(GATA)_n$ repeats in the genome of *B. napus var. oleifera* cv Topas. DNA was isolated from leaves (*a, g*), stems (*b, h*) and roots (*c, i*) of an individual plant, as well as from three individual plants regenerated from tissue culture (*d, k*, R19-1; *e, l*, R19-2; *f, m*, R19-372). Purified DNA of each sample was digested with *Msp*I or *Hpa*II, electrophoresed (5 µg per lane), and in-gel-hybridized to $(GATA)_4$. The sizes of molecular weight markers are given in kb

patterns obtained with *Msp*I and *Hpa*II (Fig. 4, lanes a–c and g–i) differed from each other, suggesting that some CCGG sites in the vicinity of $(GATA)_m$ repeats are methylated. Both *Msp*I- and *Hpa*II-generated patterns revealed some tissue specifity: the signal intensity of a small 1.2 kb fragment observed with both enzymes (i.e., probably flanked by unmethylated CCGG sites) is strong in leaves, weak in stems, and almost undetectable in roots. This result is probably the consequence of a tissue-specific methylation state.

Somaclonal variation is a phenomenon often observed in plants regenerated from tissue culture (Evans 1989). To analyze fingerprint stability and CCGG methylation in somaclonals, three protoplast regenerants derived from the same plant (cultivar Topas) were compared. While R19-1 and R19-2 looked like normal *B. napus* plants, R19-372 showed a more compact morphology. DNA was isolated from leaves of these plants, digested with *Taq*I, *Msp*I or *Hpa*II and hybridized to $(GATA)_4$ (Fig. 3, lanes o–q; Fig. 4, lanes d–f and k–m). While no differences between R19-1 and R19-2 were seen with either enzyme, R19-372 differed from the other regenerants in two minor aspects. First, the major *Taq*I-fragment around 2.3 kb appears to be somewhat smaller. Second, a band around 6.5 kb is missing in the *Hpa*II-digested sample.

Discussion

The occurrence of all six investigated simple sequence motifs in *B. napus* further substantiates the concept of the ubiquitous appearance of this DNA sequence category in eukaryotic genomes (Tautz and Renz 1984; Epplen et al. 1991; Weising et al. 1991b). Our results also support previous findings that some oligonucleotide probes are more suitable for DNA fingerprinting than others. For example, $(GATA)_4$ provides clear-cut, polymorphic banding patterns in most plant species analyzed to-date (Van Heusden et al. 1991; Van Houten et al. 1991; Weising et al. 1991a, b, 1992; Beyermann et al. 1992; Schmidt et al. 1992). Patterns created by the $(GTG)_5$ probe are also usually distinct, but appear monomorphic in many plant species (Tzuri et al. 1991; Weising et al. 1992, and unpublished results). $(CT)_8$ and $(CA)_8$, on the other hand, often produce smeared signals (Weising et al. 1991a; Beyermann et al. 1992; this study). It is as yet unclear why some simple sequence motifs are organized differently and/or are more polymorphic than others. Simple sequence repeat polymorphisms are generally thought to be created by slipped-strand mispairing during replication (Levinson and Gutman 1987). Recent in-vitro experiments have shown that slippage rates depend on the length and the sequence composition of the repeated motifs, and a positive correlation of slippage rates with the AT-content of some trinucleotide repeats was observed (Schlötterer and Tautz 1992). If the same holds true in vivo, $(GATA)_m$ repeats (AT-content of 75%) might be more prone to slippage (and thus to create polymorphism) than $(GTG)_n$ repeats (AT-content of 33%). However, additional factors are likely to influence the slippage rate in vivo since, in contrast to the situation in plants, $(GTG)_5$ reveals hypervariability in man (Schäfer et al. 1988). Solving the question of how variability is generated at the base level, will certainly require cloning and sequencing of the respective loci and their flanking regions.

A pair of isoschizomers was used to test cytosine methylation at CCGG sites in the vicinity of simple repeat stretches. The different patterns obtained by hybridization to *Hpa*II and *Msp*I, respectively, suggest that methylated cytosines occur in the vicinity of $(GATA)_m$- (Fig. 4) as well as $(GTG)_n$-stretches (data not shown). No conclusive results were obtained for the other probes, since weak signal intensities and clustering of bands in the high molecular weight range prevented the proper evaluation of *Hpa*II- and *Msp*I-generated patterns. Though it is generally assumed that repetitive DNA is highly methylated in plants (Grellet et al. 1986; Belanger and Hepburn 1990, and references cited therein), most studies on this topic performed to-date focussed on satellite DNA and rDNA. To our knowledge, this is the first report on the occurrence of cytosine methylation in the vicinity of simple repeats in the plant genome. Interestingly, we found that a particular 1.2 kb *Msp*I/*Hpa*II band was distributed in an organ-specific manner (Fig. 4). Tissue-specific methylation patterns which were correlated with gene activity were previously reported for the upstream region of plant genes [e.g., the maize zein gene (Bianchi and Viotti 1988) and a bean phytohemagglutinin gene (Riggs and Chrispeels 1990)]. However, hybridization of *Sau*3A/*Mbo*I-digested DNA of human cell populations to a human minisatellite probe showed that tandem-repetitive DNA might also be methylated in a tissue-specific manner (Berneman et al. 1989). The significance of organ- or tissue-specific methylation of repetitive DNA is not clear, but might be related to chromatin structure (Selker 1990).

Though the six tested probes revealed different levels of polymorphism, none of them is suitable for the unequivocal identification of cultivars. While fingerprints created by $(GATA)_4$ are too variable for this purpose (Fig. 2), those created by the other probes are obviously too monomorphic. Thus, the use of isozymes (Mündges et al. 1990) and RAPD markers (Hu and Quiros 1991) seems presently preferable over oligonucleotide fingerprinting for 'passporting' of *B. napus* cultivars and the protection of breeder's rights.

Other applications can, however, be recommended. First, the high distinguishing capacity of the $(GATA)_4$ probe lends itself to monitoring the effects of tissue culture on genome stability (i.e., somaclonal variation, Evans 1989). Preliminary results obtained with a limited number of somaclonals derived from the same mother plant showed slight differences in the $(GATA)_4$-fingerprint, as well as in the state of methylation of CCGG sites in the vicinity of $(GATA)_4$-stretches (Fig. 4). Second, the analysis of fingerprint patterns may assist in gene introgression programs started from a wide cross or a somatic hybrid between different species of the Brassicaceae. Multiple loci revealed by DNA fingerprints represent a set of polymorphic markers that are usually dispersed throughout the genome (Epplen et al. 1991) and may, therefore, serve as a criterion for 'similarity' between backcross progeny and the recurrent parent. The number of backcross generations is likely to be reduced by following this strategy (Hillel et al. 1990). Third, oligonucleotide probes are useful tools for the characterization of natural and artificial hybrids between different *Brassica* species. Last, but not least, the relative abundances of specific simple sequence motifs [e.g., the non-uniform distribution of $(GATA)_m$-, $(GACA)_m$- and $(CT)_p$-repeats throughout the Brassicaceae (Poulsen et al., unpublished results)], as well as conserved fingerprint patterns generated by probes such as $(GTG)_5$, might be informative with respect to the evalutionary relationship among *Brassica* species and their allies, which is still a matter of discussion (see e.g., Prakash and Hinata 1980; Quiros et al. 1988; Song et al. 1990).

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