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The detection of somaclonal variants of beet using RAPD

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Summary. Plantlets were regenerated by adventitious shoot budding in tissue culture from leaf explants of a single genotype of sugar beet. DNA was extracted from the parental plant and from 120 regenerants. RAPD analysis was carried out using five decanucleotide primers; 4,557 RAPD marker bands were examined and two polymorphisms were observed. Thirty secondary regenerants were then derived, using the same tissue culture technique, from thirty of the primary regenerants. Again RAPD analysis was employed and a single band polymorphism was observed out of 1,050 bands examined. The overall frequency of detection of somaclonal polymorphisms using RAPD (3 in 5,607 = 0.05%) is similar to frequencies previously reported using isozyme and RFLP technologies.

Abbreviations. BAP = 6-benzylaminopurine; IBA = indole-butyric acid; MS = Murashige and Skoog medium (Murashige and Skoog, 1962); RAPD = Random Amplification of Polymorphic DNA.

Introduction

The production of regenerated sugar beet plants through tissue culture, either adventitiously or from callus, is increasingly useful for a range of purposes. Their primary use is as part of a genetic transformation process involving either *Agrobacterium* or particle bombardment. For sugar beet and other cultivated beets adventitious shoot regeneration has been achieved in a number of ways and each has proved to be, to some extent, genotype-specific and to give rise to different numbers of regenerants. Such techniques have involved shoot regeneration from shoot-derived callus (Saunders and Daub 1984; Sabir et al. 1992), leaf pieces (Saunders and Shin 1986; Saunders and Doley 1986; Mikami 1989) and petiole leaf budding (Saunders 1982; Saunders and Shin 1986; Tetu et al. 1987; Freytag et al. 1988).

It is important in plant regeneration protocols to assess the level of background genetic change resulting from the tissue culture process. Tissue culture-induced genetic variation, now termed somaclonal variation, is defined as the variation that arises *de novo* during the period of dedifferentiated cell proliferation that takes place between the culture of an explant and production of regenerants. This variation has been observed among regenerants from a large number of species, and its origins, cause and application to plant breeding have been widely investigated and have formed the basis of a number of reviews (Phillips et al. 1994; Lee and Phillips 1988; Evans and Sharp 1988; Scowcroft and Larkin 1988; Karp, 1989). In two cases, the molecular mechanism underlying a phenotypic change has been elucidated (Brettell et al. 1986; Dennis et al. 1987); a number of other mutation types that are likely to be the cause of such phenotypic variation have been described including chromosome breakage, single base changes, changes in copy numbers of repeated sequences and alteration in DNA methylation patterns. A model in which some of these phenomena may be linked, involving a process related to the RIP (repeat-induced point mutation) system originally observed in *Neurospora* (Selker, 1990), has recently been described in plants (Phillips et al. 1994).

Recently, DNA-based marker techniques have been employed to detect somaclonal mutations. Such studies have usually involved searches for RFLPs and have led to reports of mutation in regenerants (e.g. Nelke et al. 1993; Kidwell and Osborn 1993) some of which have been detected within the mitochondrial genome (e.g. Deverno et al. 1994; Hartmann et al. 1994). The PCR-based molecular marker technique known as RAPD (Williams et al. 1990) has been increasingly used for estimations of genetic diversity, for varietal fingerprinting, for linkage mapping, and for the identification of somatic hybrids (Newbury and Ford-Lloyd 1993). In a few cases, RAPD technology has been used in the analysis of culture-derived material, but banding changes have only been reported among the somaclones of the two grasses *Lolium* (Wang *et al.* 1993) and *Triticum* (Brown et al. 1993). Moreover, except for experiments with *Picea* (Isabel et al. 1993), all other reported attempts to use RAPD for this purpose have been carried out using grass species (Wang et al, 1993;

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Brown et al, 1993).

In this study, we have applied RAPD technology to somaclones of the dicotyledonous crop plant, fodder beet. Previous measurements of somaclonal variation using molecular markers have tended to be assessments using single species and a single technique (but see Sabir et al. 1992). One of the intentions in this work was to compare the frequency of polymorphisms detected using RAPD with the already available frequencies detected using isozyme and RFLP (Sabir et al. 1992; Levall et al. 1994); the work would be carried out on somaclones produced from the same beet accession and using the same regeneration protocol. This would provide independent data to help assess the frequency of genetic changes within the beet genome during the tissue culture process. The comparison should also allow future workers to make a more informed choice of technology for the measurement of somaclonal variation in their test species.

Materials and Methods

Plant material Seed germination and all subsequent tissue culture was carried out at 25°C under a 16h photoperiod with a light intensity of 80 μmol $m^{-2}s^{-1}$ provided by Thorn PlusLux 3500 fluorescent lamps. Seedlings from the fodder beet accession B1064 (from the Birmingham beet collection maintained by Dr. B.V. Ford-Lloyd) were obtained by soaking seedballs in concentrated sulphuric acid for 20 min, washing three times in sterile distilled water, surface sterilising in 10% sodium hypochlorite for 15 min, washing a further three times in sterile distilled water, and placing on *Rhizobium* complete medium (yeast extract 3 gl^{-1}, tryptone 5 gl^{-1}, agar 8 gl^{-1}). After an average of 15 days, uncontaminated seedlings were transferred to MS medium containing 2% sucrose plus 0.25 mgl^{-1} BAP; in subsequent subcultures this medium and the same medium without hormone were used in alternation. Subclones of genotype (= individual) B1064/124 were used as source plants for these investigations. Whole leaves were detached and these were cultured on RV medium (Freytag et al. 1988) supplemented with 0.1 mgl^{-1} IBA and 0.25 mgl^{-1} BAP. A total of 120 adventitious shoot regenerants were obtained and these were subjected to RAPD analysis. Subsequently, leaves of 30 of these primary regenerants were used as explants to derive 30 secondary shoot regenerants and these were also subjected to RAPD analysis.

DNA preparation:: DNA was extracted from fresh leaf tissue (10-100mg) following the minipreparation method of Dellaporta et al. (1983). DNA concentration was assessed by measuring fluorescence after binding to Hoechst 33258 dye in a TKO 100 minifluorometer following the manufacturer's instructions. DNA samples were stored at -20C before use.

RAPD analysis: Polymerase chain reactions were carried out in 50 μl volumes containing 0.1 μg of DNA, 1 unit of Taq polymerase and 1 x reaction buffer (Boehringer/Mannheim), 200 μM of each dNTP, 2.5 mM $MgCl_2$ and 0.1 pmol of decanucleotide primer. The reaction mixes were overlaid with mineral oil and amplification was carried out as described previously (Munthali et al. 1991) using a Hybaid HB TR1 thermal cycler. Twenty μl of the reaction mix was then subjected to electrophoresis in a 1.0% (w/v) agarose gel, the DNA bands visualised by ethidium bromide staining (Sambrook et al. 1989), and photographs taken using a Polaroid system. The decanucleotide primers were produced by Alta BioScience (Birmingham University) and the sequences are given in Table 1.

Table 1. The decanucleotide primers of arbitrary sequence used in this study.

Primer number	Sequence
1	CCCACAGTCA
2	CTCGCTGTCG
3	GGCGTATGCG
4	GACGAGTACG
5	GTGCGTATGG

Results

A total of 120 shoot cultures were regenerated by adventitious budding from whole leaf explants of subclonal material of a single genotype (individual) of the fodder beet accession B1064. This accession was selected because it had previously been shown to produce adventitious shoots from callus cultures (Sabir et al. 1992; Sabir and Ford-Lloyd 1991). The individual genotype B1064-124 was selected because it produced shoots more prolifically than other genotypes tested from this accession (data not shown).

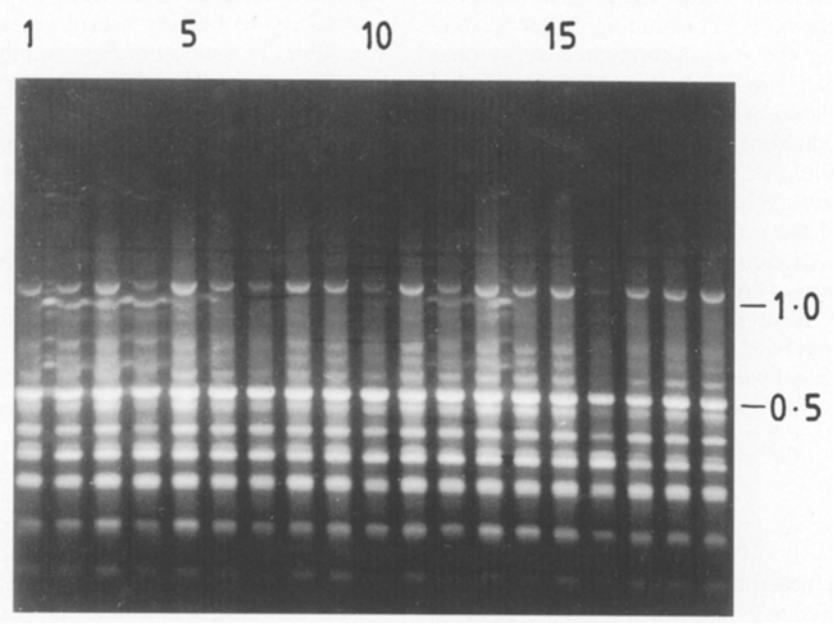

Fig. 1 Agarose gel electrophoresis of amplified sequences from a RAPD reaction directed by primer 2 using DNA extracted from 19 beet regenerants (lanes 1 - 19). The mobilities of the 1.0 and 0.5 kbp size markers are indicated.

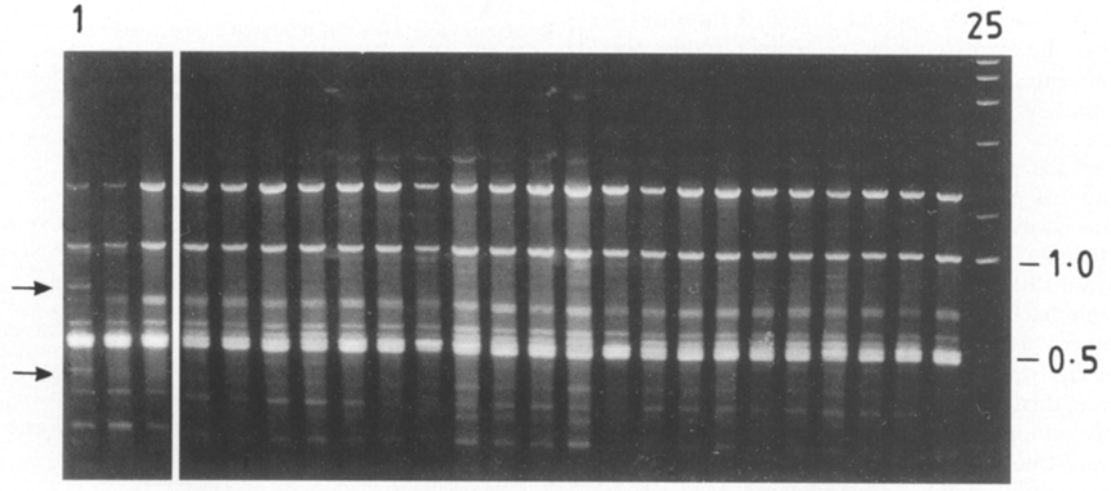

Fig. 2 Agarose gel electrophoresis of amplified sequences from a RAPD reaction directed by primer 5 using DNA extracted from 24 beet regenerants (lanes 1 - 24). Two novel bands are indicated by arrows in lane 1. Molecular weight markers are present in lane 25 and mobilities of the 1.0 and 0.5 kbp size markers are indicated.

DNA was isolated from all of the regenerated shoots, the concentrations were estimated, and the samples were used for RAPD analysis using arbitrary decanucleotides to direct amplification. Following electrophoresis, the DNA banding patterns were photographed. The lack of variation in most banding profiles is illustrated in Fig. 1. Five primers were used in this analysis yielding an average of 7.9 scorable bands. The total number of bands scored (number of regenerants x number of bands with all five primers) was 4,557. Of these, all were identical to those observed in the B1064-124 mother shoot clone except for two novel bands (of approximately 1.4 and 0.8 kb) produced during the analysis of regenerant 11 using primer 5 (Fig. 2). Independent repeat DNA extractions were performed on this regenerant and the novel bands were again observed using this primer. Since no RAPD banding differences had been detected among the micropropagated sub-clonal progeny of individual B1064/124, the variation revealed among regenerated shoots was related to the process of adventitious shoot production.

As a futher assessment of variation frequency on the same genotype, second cycle regenerants were produced from each of thirty of the primary regenerants and again RAPD analysis was carried out using extracted DNA. Once more five primers were employed and this time a total of 1,050 bands were scored. A novel band was observed in second cycle regenerant number 14 using primer 9 (Fig. 3); the band was observed again using DNA obtained following a repeat extraction from this regenerant.

Discussion

Previous authors have noted the differential tissue culture responses between different genotypes of outbreeding crops in general (Atanassov 1986) and specifically within genotypes of beet from a single accession (Bhat 1986; Saunders and Daub 1984; Sabir and Ford-Lloyd 1991). The technique of shoot regeneration by adventitious budding in beet has previously been reported (Freytag et al. 1988; Saunders 1982; Saunders and Shin 1986; Tetu et al. 1987; Sabir and Ford-Lloyd 1991) and has proved to be much more applicable to a wide range of beet genotypes than other techniques regeneration described.

Previous work in our laboratory (Sabir et al. 1992) has allowed the assessment of the frequency of somaclonal variation in beet regenerants using isozyme and RFLP markers. The number of isozyme alleles examined was calculated as the number of regenerants (764) x the number of enzyme loci (8) x the ploidy level (2) = 12,224. Since seven polymorphisms were observed, the frequency of variant alleles was calculated as 0.06%. The frequency of variant alleles assessed using RFLP was 0.1%; this figure is likely to be less useful since it was based upon the analysis of a subset of regenerants which had been selected because they showed isozymic or morphological variation. In a separate and more extensive RFLP study, Levall et al. (1994) assessed the frequency of variant alleles in beet plants regenerated from UV light-treated callus. Using 50 probes and 42 regenerants they were able to examine 7,644 alleles; the three polymorphisms detected arose at a frequency of 0.03%. Taking a general frequency of marker polymorphism in regenerated beet of 0.05%, it can be calculated that to be 95% confident of detecting such an event one would need to examine 5,990 marker isozyme or RFLP bands. However, both isozyme and RFLP markers are co-dominant (i.e. the techniques allow the detection of two alleles at a heterozygous locus in a diploid). By contrast, RAPD markers are usually dominant (Williams et al. 1990; Newbury and Ford-Lloyd 1993). It is generally accepted that alleles tend to either support amplification of a genomic sequence or they do not, so that the occurrence of a specific band does not allow the discrimination between homo- and heterozygotes. This characteristic of RAPD can be predicted to have no influence on its effectiveness for revealing novel bands, resulting from mutant alleles now able to support amplification of a

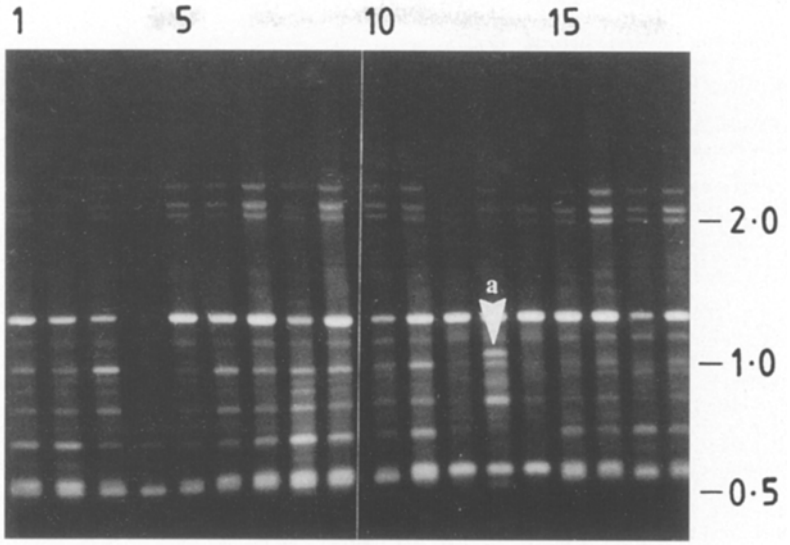

Fig. 3 Agarose gel electrophoresis of amplified sequences from a RAPD reaction directed by primer 9 using DNA extracted from 18 beet regenerants (lanes 1 - 18). A novel band in lane 13 is indicated by an arrow. The mobilities of the 2.0, 1.0 and 0.5 kbp size markers are indicated.

sequence. However, the dominant nature of the bands means that at a homozygous locus a mutant allele no longer able to support amplification of a marker band will be masked in a diploid. Hence, one might expect the efficiency of the RAPD method to be lower at detecting somaclonal variation than isozyme and RFLP techniques.

Some other workers have attempted to detect somaclonal variation using RAPD. In RAPD analyses of *Lolium* and *Festuca* regenerants, Valles et al. (1993) found no variants, whereas polymorphisms were detected in *Lolium* by Wang et al. (1993). Brown et al. (1993) reported RAPD polymorphisms in *Triticum* regenerants. In none of these cases was the total number of markers scored reported. Isabel et al. (1993) detected no polymorphisms in *Picea* regenerants after scoring a total of 900 bands (10 selected markers for 90 somatic embryos). The total number of bands scored may not have been sufficiently high to allow detection of polymorphisms if these were present at a frequency close to 0.05%. Also, in this study only ten specific bands were selected for scoring following pre-screening of parental material. The lack of scoring of novel bands would lead to an underestimation of the frequency of polymorphism. Devaux et al. (1993) carried out a large scale investigation into somaclonal variation in *Hordeum* dihaploids using both RFLP and RAPD analyses. They scored 273 RFLP and 89 RAPD markers for 60 plants (total scored for RFLP = 16,380, and for RAPD = 5,340). No polymorphisms were detected using either technique. This suggests that for this embryogenically-derived material the frequency of polymorphism was much lower than 0.05%.

In our study, DNA was extracted from the parental plant and initially from 120 regenerants. RAPD analysis was carried out using five decanucleotide primers and from a total of 4,557 RAPD bands examined two polymorphisms were observed (a frequency of 0.04%). Thirty secondary regenerants were then derived, using the same tissue culture technique, from 30 of the primary regenerants. RAPD analysis revealed a single band polymorphism out of 1,050 examined (a frequency of 0.1%). The overall frequency was thus three polymorphisms in 5,607 bands (= 0.05%). Despite the dominance of RAPD markers, the frequency of detection of somaclonal polymorphisms in beet using RAPD is therefore very similar to frequencies previously reported using isozyme and RFLP technologies. Given that marker information can be obtained using RAPD very quickly, that the amount of starting material required is very small, that a very large number of non-species specific primers is commercially available, and that RAPD markers can be cloned and converted to RFLP probes, RAPD methodology, along with other PCR-based technologies, has been shown to be a very effective means of detecting somaclonal variation in comparison to other techniques.

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