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Determination of genetic stability using isozymes and RFLPs in beet plants regenerated in vitro

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Summary. Sugar, fodder and garden beet (*Beta vulgaris* L.) plants have been regenerated in culture from a range of explant material. Of the regenerants 764 were subjected to isozyme analysis using eight enzyme-specific stains, and 60 were subjected to RFLP analysis using three cDNA probes. Both molecular techniques allowed the identification of somaclonal variant plants. Assessment of the numbers of variant isozymes and restriction fragments has allowed the calculation of the approximate percentage of variant alleles occurring in any one somaclonal regenerant, namely between 0.05% and 0.1%.

Key words: Beet – Somaclonal variation – Isozyme – RFLP – Genetic stability

Introduction

We have attempted to monitor the degree of genetic instability in different cultures of beets producing shoots de novo. Such monitoring is only realistic with this material when a precise description of the parental genotype can be provided with which any potential genetic variants can be compared. This is most easily achieved using isozyme or RFLP analysis, particularly where the material is highly genetically variable and allogamous, as is the case with beet (Atanassov 1986; Nagamine et al. 1989a). It is generally assumed that genetic changes arising as a result of tissue culture may be caused by changes in chromosome number or structure, DNA amplification, activation of transposable elements and cytoplasmic genome rearrangements, which will give rise to various alterations in the amount or organisation of genomic DNA (Larkin et al. 1989). Some changes to genes cannot be observed at a morphological or physiological level because the structural difference in the gene product may not alter its biological activity sufficiently to result in an altered phenotype. 'Silent' mutations at morphological or physiological levels are significant since they allow an estimation of the frequency of genomic change as a result of in vitro culture. In this paper we describe the use of isozyme and RFLP techniques to calculate the rate of culture-induced genetic variation in beet.

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Materials and methods

Production of regenerants

Methods for the de novo regeneration of plants from shoot culture callus, from petiole explants, from leaf discs and from leaf-like tissue explants have already been described, as have those for the production of micropropagants from axillary shoots (Sabir and Ford-Lloyd 1991). The sources of parental genotypes, regeneration methods and number of regenerants analysed using isozymes and RFLPs are given in Table 1.

Considerable effort was made to standardise conditions of sampling in terms of actual and physiological age of leaves. However, complete standardisation was impossible because of the length of time for regeneration from tissue culture and subsequent rooting. As a result, the potted plants from which leaf samples were taken fell within an age range of 3 months.

Isozyme analysis

A 40-mg sample of plant tissue of a fully developed leaf, normally fourth from the apex, was taken from plants growing in a glasshouse (18°–25 °C, 16 h light). Samples were macerated with 65 µl of extraction buffer (0.05 *M* TRIS-HCl pH 6.8, 2% mercaptoethanol, 2% PVP, 2% PVPP and 0.03% ascorbic acid) plus acid-washed sand. The extract was then absorbed onto a small filter paper wick, and starch gel electrophoresis (SGE) was performed as described by Nagamine et al. (1989a).

With buffer system A (Van Geyt and Smed 1984) malate dehydrogenase (MDH) and shikimate dehydrogenase (SKD) were assayed according to Wendel and Weeden (1989), and

Table 1. Parental material (all diploid) and numbers and origins of somaclones studied

Cultivar and genotype	Regeneration method	Number of plants used for analysis	
		Isozymes	RFLPs
Garden beet			
Piatta d'Egitto (B791-9)	Petiole budding	26	–
Fodder beet			
White Knight (B1064-102)	Shoot culture callus	19	–
Sugar beet			
Male sterile line (B814-2)	Leaf-like tissue	98	18
Sharpe's Klein E (B1085-11)	Shoot culture callus	462	20
	Leaf disc callus	15	12
	Petiole budding	144	10

phosphoglucomutase (PGM) was assayed according to Tanksley and Orton (1983). With buffer system B (Shaw and Prasad 1970) glucose phosphate isomerase (GPI) and 6-phosphogluconate dehydrogenase (6-PGD) were analysed according to Golding et al (1985) and Shaw and Prasad (1970). With buffer system C (Shaw and Prasad 1970) glutamate oxaloacetate transaminase (GOT) and glutamate dehydrogenase (GDH) were assayed according to Brown et al. (1978) and Shaw and Prasad (1970). Electrophoresis was carried out initially at either 35 mA (for A and B) or 50 mA (for C) for 0.5 h when wicks were removed, and then at either 25 mA (for A), 30 mA (for B) or 40 mA (for C) for 5 h.

Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) was assessed using four cDNA sequences to probe *Hin*dIII-digested DNA extracted from 60 regenerant plants. These plants were chosen at random or because they showed some morphological or isozyme variation when compared to parental genotypes.

DNA was isolated using the procedure of Dellaporta et al. (1983). Digestion with *Hin*dIII and Southern (1975) transfers were effected according to Nagamine et al. (1989b). One cDNA insert (L24) had already proved useful as a hybridisation probe (Nagamine et al. 1989b), and two others were selected randomly for use. Amplification of DNA inserts was carried out using the polymerase chain reaction (Güssow and Clackson 1989). Individual plaques were transferred to 50 µl water and incubated at room temperature for 30 min with occasional agitation. Five µl of the bathing solution was added to the GeneAmp reaction mixture, which was made up according to the manufacturer's (Perkin Elmer Cetus) instructions. The mix contained 22- and 24-base oligomers that flank the insertion site in λgt10 (sequences below), and the annealing temperature in the PCR programme was 49°:

GT10FP: $^{5'}$GAGCAAGTTCAGCCTGGTTAAG$^{3'}$

GT10RP: $^{3'}$TGGGACCTTCTTTATGAGTATTCG$^{5'}$

Amplified DNA fragments were purified using a low-melting-point agarose (Sigma) gel. cDNA inserts were excised from the gels and labelled using ^{32}P-dCTP (Amersham Int) in the random hexanucleotide primer-directed synthesis method of Feinberg and Vogelstein (1983, 1984). Hybridisation was carried out as described by Nagamine et al. (1989b).

Results

Isozyme analyses

Preliminary experiments to examine the effects of leaf position and age indicated that satisfactory enzyme activity was provided by the youngest fully developed leaf. No variation in isozyme banding pattern was ever found to be attributable either to age of leaf or age of plant (within the age range of the material).

A parental phenotype could be defined for each of the genotypes which had been the subject of adventitious regeneration in relation to each of the enzymes analysed. The vast majority of somaclones also conformed to these parental phenotypes. It should be noted that no variants of the parental phenotypes were detected after initial in vitro micropropagation of these genotypes. However, a small number of somaclones deviated from these parental types for some of the enzymes. No somaclonal variation was found in the isozymes of SKD, PGM, or GOT. However, of the isozymes of MDH, GPI, IDH, PGD and GDH a total of seven variants were identified.

GPI – Normal parental phenotypes, together with a somaclonal variant phenotype (BSC-68) of a regenerant from shoot culture-derived callus are shown in Fig. 1. The aberrant phenotype shows considerably reduced activity in two bands, with an increase in activity in the third.

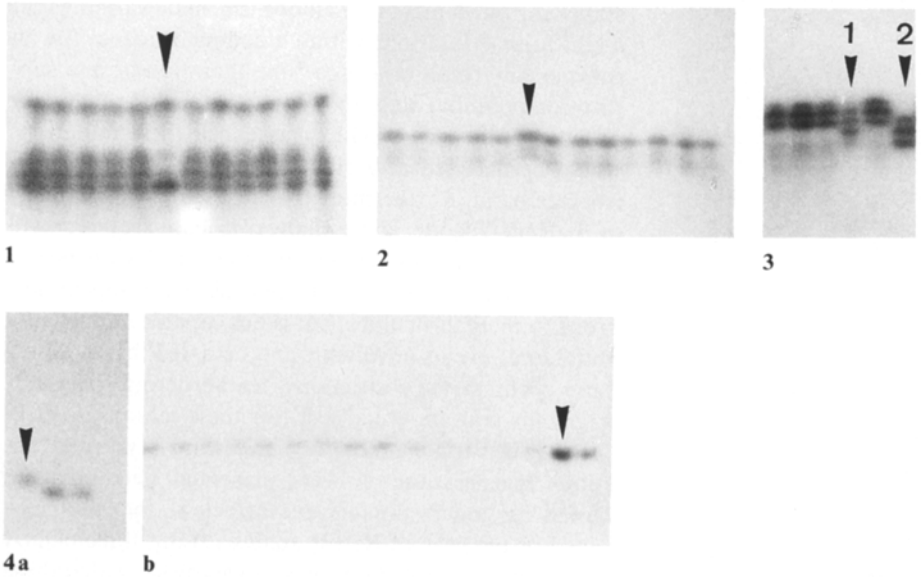
PGD – Normal parental phenotypes, together with a somaclonal variant phenotype (N-103) of a regenerant from leaf-like tissue are shown in Fig. 2. The somaclonal variant is identifiable because of consistently faster migration of both bands during electrophoresis.

IDH – Normal parental phenotypes, together with two somaclonal variant phenotypes (BSC-9 and BSC-68) are shown in Fig. 3. Both variants are derived from shoot culture callus from parental material whose phenotype is represented by B1085-11 (Fig. 3). Different slower migrating bands, probably representing allelic mutations, are present in these somaclonal variants.

GDH – Normal parental phenotypes, together with two somaclonal variant phenotypes are shown in Fig. 4a and b. Both variants are regenerants from shoot culture callus from the parent B1085-11. One variant is represented by a faster migrating band (4a), while the other variant shows a band that is fractionally slower migrating and has consistently much higher activity (4b).

RFLP analysis

Probe L24 (approximately 0.7 kb) hybridised with ten fragments in the parental genotype B1085-11, and this pattern was repeated for the majority of somaclones as illustrated in Fig. 5a–c. Three somaclonal variants of this pattern were identified. In Fig. 5a it can be seen that somaclone BSLD-13 (from leaf disc regeneration) has



Figs. 1–4. Isozyme analyses. **Fig. 1.** GPI zymogram for genotype B1085-11. *Arrow* points to the somaclonal variant BSC-68 derived from shoot-culture callus. **Fig. 2.** PGD zymogram for genotype B814-2. *Arrow* points to the somaclonal variant N-103 derived from leaf-like regenerant. **Fig. 3.** IDH zymogram for genotype B1085-11. *Arrows* point to the somaclonal variants BSC-9 (*1*) and BSC-68 (*2*) generated from shoot-culture callus. **Fig. 4a, b.** GDH zymograms for genotype B1085-11. **a** *Arrow* points to the somaclonal variant BSC-9, derived from shoot-culture callus. **b** *Arrow* indicates the somaclonal variant BSC-139, derived from shoot-culture callus.

lost two fragments (4.5 and 5.8 kb), and in Fig. 5b it is evident that somaclone BSC-9 (from shoot culture callus) has lost one fragment (12 kb). The somaclonal variant BSC-68 (from shoot culture callus), also derived from the parental genotype B1085-11, exhibited extreme variation in that only five fragments hybridised to this probe (Fig. 5c).

Probe L25 (approximately 0.6 kb) hybridised with only one fragment in all of the material examined, either parental genotypes or somaclones, with the exception of the somaclone BSC-68 derived from shoot culture callus of the parental genotpye B1085-11. This variant was characterised by having an additional fragment of 5.5 kb (Fig. 6).

Probe L27, approximately 3.2 kb in length, normally hybridised with three fragments in somaclones derived from, and material of, parental genotype B814-2. One somaclonal variant was detected. N-92 showed an extra fragment corresponding to 7.8 kb (Fig. 7).

Morphological variation

Phenotypic variation was only clearly observed in plants regenerated from two genotypes (B1085-11 and B814-2). The most obvious variation was in fusion of petioles between leaves. Other variations in leaf shape, leaf serration and leaf venation could be observed. This variation was not uncommon and occurred at a frequency much greater than that of isozyme or RFLP variants.

Discussion

It is assumed that both the variant isozymic and RFLP phenotypes represent genetic changes associated with somaclonal variation in adventitious regenerants, and therefore can be used to provide estimates for the amount of genetic change that can be expected to result from systems of in vitro adventitious regeneration in beet. In a simple calculation based upon the observed isoenzyme variation, it is possible to extrapolate the frequency of genetic change giving rise to somaclonal variation. The assumption has to be made that each isoenzyme variant is a result of allelic variation caused by a single mutational event. Thus, there were:

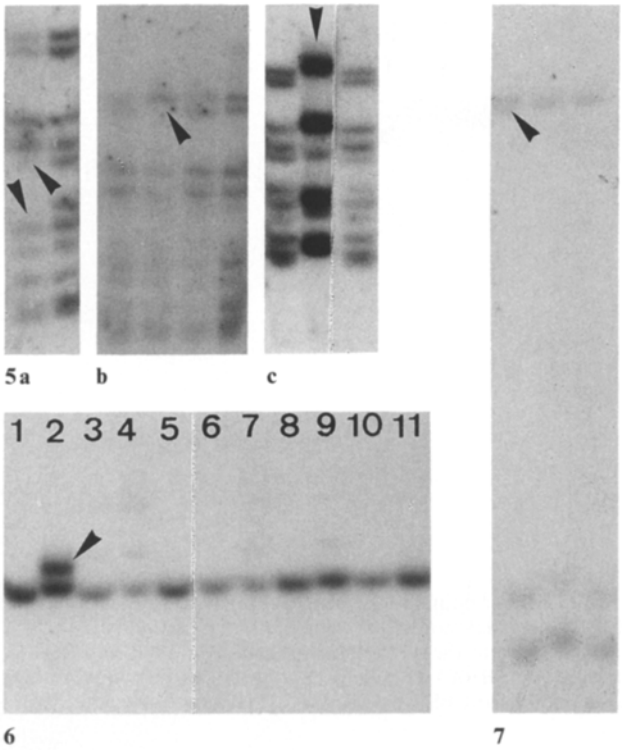
764 plants analysed
for eight enzymes
for 16 alleles (assuming all regenerants are diploid).

Total number of alleles examined = 12,224.

Seven allelic variants were observed.

Percentage of variant alleles = 0.057.

From the known DNA content of the beet genome [estimated to be 1.2 picograms (Bennett and Smith 1976)], and making the assumption (Grierson and Covey 1988) that only 4% of the genome may be transcribed to protein as well as assuming an average polypeptide size (350 amino acids), we are able to calculate that there are approximately 88,000 genes in the diploid beet genome.



Figs. 5–7. RFLP analysis. **Fig. 5a–c.** RFLP patterns resulting from the hybridisation of cDNA clone L24 to *Hin*dIII-digested total genomic DNA extracted from B1085-11 leaf disc callus-derived (*a*), or shoot culture callus-derived (**a** and **c**) somaclones (BSLD-13, BSC-9 and BSC-68, respectively). (*Arrows* indicate differences in banding pattern compared to parental). **Fig. 6.** RFLP patterns resulting from hybridisation of cDNA clone L25 to *Hin*dIII-digested total genomic DNA. DNA was extracted from leaf tissue of plants regenerated in vitro from sugar beet genotype B1085-11 shoot-culture callus (*tracks 1–5*) and adventitious budding on leaf petiole (*tracks 6–11*). The *arrow* indicates the change in RFLP pattern of the variant BSC-68. **Fig. 7.** RFLP patterns resulting from hybridisation of cDNA clone L27 to *Hin*dIII-digested total genomic DNA extracted from B814-2 somaclones. *Arrow* indicates the position of the extra DNA fragment of variant N-29

Based on this figure, and on the calculated percentage of variant alleles due to somaclonal variation (above), it may be estimated that as many as 50 genes may be altered in any one somaclonal regenerant plant from beet tissue culture.

Calculations made from the RFLP analysis were possibly biased because the small number of regenerants studied by this method were deliberately selected on the basis that they had already shown isoenzyme variation, or were morphologically aberrant. If this is ignored, and a calculation is made regardless, then a figure rather higher than that above, but in the same order of magnitude, is reached, namely 0.1% or as many as 80 genes per diploid regenerant.

Although isozyme and RFLP techniques have allowed the identification of variant regenerants, neither method allows for the detection of all of the genetic variants that might arise. Mutations within a coding sequence for an enzyme may result in a slight size alteration or in a substitution of amino acids that does not alter the charge or activity of the gene product; neither of these mutations would be detected as a change in migration rate of an isozyme band. Furthermore, because of the redundancy of the genetic code, some changes in base sequence will not be expressed as changes in amino acid sequence of the encoded protein. RFLP analysis may appear less prone to these difficulties, but is not capable of detecting mutational events involving one or a few bases unless these occur at the restriction sites bordering the DNA fragments that are visualised. For these reasons, the frequencies of variant production following in vitro adventitious regeneration of beets presented here must be viewed as underestimates. Nevertheless, the data, obtained in the case of RFLP analysis using three cDNA probes and one restriction enzyme clearly show that these molecular techniques can be used to follow somaclonal variation in beet.

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