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A rapid screening technique for the detection of repeated DNA sequences in plant tissues

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Summary. DNA sequences cloned from nuclear and mitochondrial chromosomes have been used as hybridisation probes to distinguish different plant genotypes. The probes are hybridised to squashed segments of tissue e.g. root tips. The 'squash-dot' method is rapid and suitable for screening large numbers of individual plants. One probe, specific for a rye repeated sequence family, enables rye chromosomes to be detected in wheat plants. A probe for ribosomal DNA enables plants with high or low numbers of ribosomal RNA genes to be distinguished. A maize mitochondrial DNA probe is used to distinguish plants with N, T or S cytoplasms.

Key words: Repeated sequences – Ribosomal DNA – Mitochondrial DNA – Plant breeding

Introduction

In plant genetics and breeding programmes methods which lead to the rapid identification of specific genotypes are usually valuable because they save time and labour. The detection of protein variants by electrophoresis or isoelectric focussing is one such method (Payne et al. 1981). Another is the detection of dwarfing genes in wheat by the application of gibberellic acid to the seedlings (Gale and Gregory 1977).

Specific DNA sequences, purified by molecular cloning, also provide the means of analysing genetic variation in complex genomes. To use the sequences, the DNA is usually radioactively labelled and hybridised to DNA purified from the organisms to be assayed. However, when large numbers of individuals are involved, as is usually the case in plant genetics and breeding programmes, purifying large numbers of DNA samples is laborious and expensive. There is therefore a need to develop, where possible, rapid methods which do not involve purifying DNA. Where a similar need occurs for bacteria then the hybridisation is conveniently carried out, as described by Grunstein and Hogness (1975) using crude cell lysates, the cells being lysed directly on to the nitrocellulose on which the bacterial colony was growing.

In this paper a modification of the Grunstein-Hogness procedure is described which enables specific DNAs to be detected rapidly in extracts squashed from plant tissue. The detection of nuclear and mitochondrial DNAs is described and its quantitative accuracy assessed. The 'squash dot' technique is particularly valuable in genetics and breeding programmes where large numbers of plants need to be assayed (Flavell 1982; Flavell et al. 1983).

Materials and methods

1 Plant materials

The following plant stocks were used:

a) Secale cereale cv. 'UC90'; *Triticum durum* cv. 'Cocorit'; wheat-rye amphiploid – 'Cocorit' × UC90'; wheat-rye addition line – Holdfast/King II chromosome 1R.

b) Triticum aestivum cv. 'Chinese Spring' and its aneuploid stocks (Sears 1954).

c) Zea mays Wf9 carrying the T, S or N cytoplasm.

2 Preparation of filters

a) Root-tips. Seeds were germinated at 24 °C until the roots were approximately 1.0–1.5 cm long. The meristematic root-tip was cut off at about 3 mm and was squashed using a metal rod onto either nitrocellulose or Whatman 541 filter paper.

b) Sections. Frozen sections of plant tissue were obtained using the method described by Gahan et al. (1967). This technique is applicable to a wide range of plant tissues. In the experiment described here, seeds were soaked in water for 1 h before dissecting out the embryos. These were then treated with polyvinyl alcohol as described and sectioned in ice at −30 °C using a Slee cryostat. The sections were mounted on wet nitrocellulose filters and allowed to dry at room temperature prior to denaturation.

3 Denaturation of DNA on filters

The DNA of the plant tissues on the filters was denatured in 0.5 M NaOH for 5 min followed by neutralisation in 1 M Tris-HCl pH 7.5 for 5 min twice and then in 1.5 M NaCl, 0.5 Tris-HCl pH 7.5 for 5 min twice. This is similar to the method for denaturing bacterial colonies on filters described by Grunstein and Hogness (1975). Filters were baked under vacuum at 80 °C for 2 h and can be stored in sealed plastic bags at −20 °C until required.

4 Probes

The ^{32}P labelled probes were prepared by nick translation (Rigby et al. 1977). The chimaeric plasmids used as probes are described in Table 1.

5 Hybridisation

Filters were first wetted by floating on water, 2×SSC (SSC = 0.15 M sodium chloride, 0.015 M sodium citrate) and 5×SSC for 5 min each. The filters were then pre-hybridised in sealed plastic bags at 60 °C for 12 to 16 h in 5×SSC, 5×Denhardt's solution (Denhardt 1966), 30 mM $NaPO_4$ pH 6.5 and denatured sonicated salmon sperm DNA (200 µg/ml). After pre-hybridisation, the radioactive probes were denatured by boiling and injected into the hybridisation bags. The bags were sealed and hybridisation was carried out at 60 °C for 16 h. After hybridisation, the filters were washed with 2×SSC, 1×SSC and 0.5×SSC all at 60 °C for 1 h or more per wash. Filters were dried at 80 °C under vacuum for 2 h.

6 Detection of hybridisation

The extent of hybridisation was estimated visually by exposing the autoradiograph to X-ray film.

To gain quantitative estimates of the extent of hybridisation the filters were cut up into strips, each strip comprising the three root-tips taken from one seedling. If necessary the squashed root-tips could be located by illuminating the filters with UV light (Gergen et al. 1979). The radioactivity of each filter strip was measured by scintillation counting.

Results and discussion

1 Detection of a highly repetitive DNA sequence from rye in squashed root tips of rye, wheat-rye hybrids and a wheat-rye addition line

Rye contains over 10^6 copies of a '480' base pair repeated sequence which is not detectable as a repeated sequence in wheat DNA by hybridisation analysis to highly purified DNA (Bedbrook et al. 1980; Jones and Flavell 1982). The sequences are present on tandem arrays on all rye chromosomes. A plasmid containing part of the repeat, pSc74, is therefore a very convenient probe to detect the presence of rye chromosomes in wheat-rye hybrids (Hutchinson et al. 1980). To investigate the suitability of crude root-tip squashes as sources of DNA for hybridisation to the probe, 3 mm root tips of rye, the rye×wheat amphiploid and a wheat line carrying a single rye chromosome were squashed on the filters, denatured and baked at 80 °C. ^{32}P labelled pSc74 DNA was hybridised to the filter paper and the extent of hybridisation detected by autoradiography. The autoradiograph (Fig. 1) shows strong hybridisation of the probe to the DNA of the rye root-tips, but not to the wheat material. The probe also hybridised strongly to the DNA of the wheat-rye amphiploid which contains both the wheat and rye genomes, but showed a reduced level of hybridisation to a wheat-rye addition line which contains the whole genome of wheat together with just one pair of chromosomes from rye (see Fig. 1). This therefore demonstrates that by hybridising a ^{32}P labelled probe to plant tissue, it is possible to detect specific repetitive chromosomal DNA sequences.

The ability to detect the presence of a single rye chromosome in wheat makes the assay suitable for detecting the presence of the short arm of rye chromosome 1R in many European wheats. This segment of rye replacing the short arm of wheat chromosome 1B has been maintained in many European wheat breeding programmes because it contains disease resistance genes (Mettin et al. 1973). However, many

Table 1

Probe	Plant DNA sequence	Plasmid vector	Reference
pSc74	Rye 480 bp highly repetitive sequence	pBR322	Bedbrook et al. (1980)
pTa71	Wheat 18S+28S ribosomal gene sequence+spacer	pACYC184	Gerlach and Bedbrook (1979)
pZms21	Maize mitochondrial sequence	pBR322	Thompson et al. (1980)

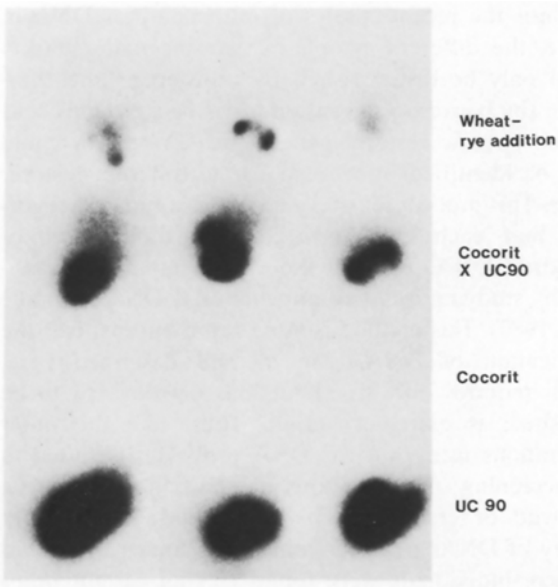

Fig. 1. Detection of rye chromosomes using a rye repeated sequence probe. Root tip segments were squashed on to filter paper, the DNA denatured and hybridised to pSc 74 (^{32}P labelled) and the hybridisation detected by autoradiography on X-ray film

breeders would prefer to select against the translocation since it is believed to have adverse effects on other agronomic characters. The 'squash dot' assay described here could be used to screen rapidly the progeny from crosses to distinguish those plants possessing the 1R segment from those with a complete wheat 1B chromosome.

The reduced hybridisation to the wheat-rye addition line indicates that the degree of hybridisation is quantitative, and depends upon the amount of the sequence present in the DNA of the plant material. This was investigated further using a different repetitive sequence, that coding for the ribosomal RNAs.

2 Hybridisation of ribosomal DNA to DNA squashed from root tips

The hexaploid wheat cultivar 'Chinese Spring' has approximately 9,150 copies of the ribosomal RNA (rRNA) gene per 2C nucleus. The numbers of rRNA genes in aneuploid stocks of 'Chinese Spring' in which the nucleolar organising chromosomes have been added or deleted have also been estimated (Flavell and Smith 1974; Flavell and O'Dell 1976, 1979). These stocks therefore provide an opportunity for examining the relationship between the amount of hybridisation of a ^{32}P-labelled probe to a root tip squash and the number of copies of a sequence present in the genome.

In any investigation of the quantitative aspects of DNA hybridisation it is important to ensure that the amount of plant material (DNA) of each genotype probed is the same. It is therefore undesirable to use roots or tissue segments which differ markedly in size since it is likely that the DNA content of the tissue will also vary. The root-tips of the aneuploid stocks probed here with the cloned ribosomal RNA gene sequence pTa71 (Gerlach and Bedbrook 1979) appeared reasonably uniform in size. However, to account for any minor differences, or any variation in the lengths of the root-tips which were cut off and squashed onto the filters, each genotype was represented by 5 replicates, each consisting of three root-tips. Within each replicate, the genotypes were completely randomised on the filter paper. An estimate of the number of counts of the ^{32}P labelled pTa71 probe hybridised to each group of three roots was made by scintillation counting. These data were then compared with the published gene numbers for each aneuploid line.

Polynomial regression analysis showed that although there was a highly significant linear component of regression, ($P > 0.01$) the quadratic component was also significant ($P > 0.05$), while the cubic and quartic components were not significant. Thus, as the gene copy number increases, so the number of cpm of probe hybridised increases rapidly but tails off as the gene copy number becomes high (Fig. 2). This failure to hybridise larger amounts of rDNA to squashes of plants with a high number of rRNA genes has not been investigated. However, it appears that within the range of 3,000 to 10,000 rRNA gene copies, it is possible to determine the ranking order of different genotypes (Fig. 2) and thus to select plants with high or low

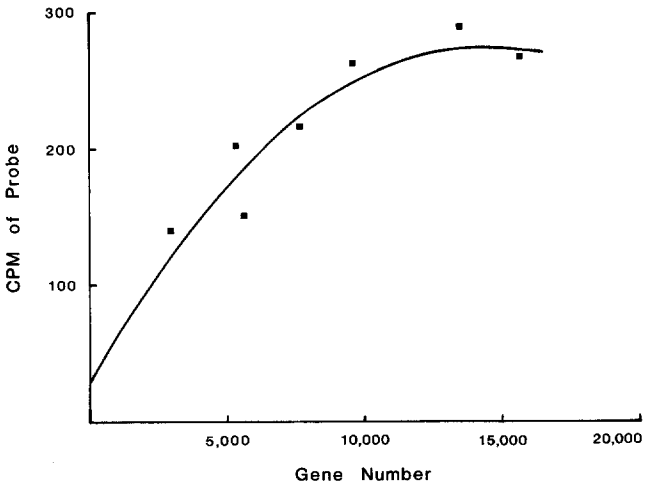

Fig. 2. Hybridisation of rDNA to squashed wheat root tips differing in the number of rRNA genes. The results (cpm of ^{32}P labelled pTa 71 hybridised to root tip material) are the means of five replicates of each genotype, each replicate consisting of three root tips. A quadratic polynomial was used to fit the curve to the data points

numbers of rRNA genes or other similarly repetitive sequences.

This technique also appears useful for estimating relative sequence copy numbers, where the copy number of the sequence in one or more genotypes is already known. It must be emphasised however that, as pointed out above, the number of cells, i.e. amounts of tissue, must be similar for each genotype to be probed.

In the two experiments described above, the copy numbers per nucleus of the probed sequences were relatively high, being in excess of 3,100 copies per 2C nucleus in the case of the ribosomal RNA genes. However, a hundred copies or fewer are also easily detected using these methods by increasing the specific activity of the DNA probe and the autoradiography time (unpublished results).

3 Detection of different mitochondrial DNAs in maize

The tissue 'squash dot' technique has also been used to detect the differential concentration of specific mitochondrial DNA sequences in maize lines possessing different cytoplasms viz. N, cms S and cms T (Duvick 1965).

Embryo sections of these three lines squashed on to nitrocellulose were probed with the clone pZms21 (Table 1). This clone shows homology to both the S_1 and S_2 sequences which exist in short linear molecules in high concentration in cms S mitochondria, but which are integrated in large chromosomes and are in much lower concentration in the mitochondrial genome of plants with N cytoplasm (Lonsdale et al. 1981). Cms T mitochondrial DNA carries deletions for a large portion of these sequences (Pring et al. 1977; Pring and Levings 1978; Thompson et al. 1980). Hybridisation of this probe to tissue sections demonstrated the quantitative difference in S element sequences for these three cytoplasms as shown in Fig. 3. Thus this technique can be utilized to distinguish the different cytoplasms without the use of genetic crossing.

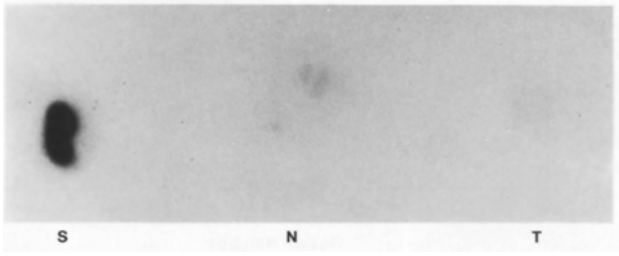

Fig. 3. Detection of different maize cytoplasms (N, S and T) by hybridisation with a mitochondrial DNA sequence. Pairs of frozen embryo sections of each genotype were dried on to nitrocellulose and hybridised with pZms 21 ^{32}P labelled DNA. The extent of hybridisation was revealed by autoradiography on X-ray film

Until the recent studies of mitochondrial DNA in maize, the different cytoplasms causing male sterility could only be distinguished by transferring the cytoplasm (by backcrossing) into nuclear backgrounds containing specific restorer genes. Each cytoplasm could then be identified by its response to different restorer genes. This procedure is very time consuming. Recognition that each cytoplasm contains different mitochondrial DNAs enabled the cytoplasms to be classified by studying isolated mitochondrial DNA (Kemble et al. 1980). This method is more rapid but involves the purification of DNA. The method described here, which requires only tissue sections or root tips to be squashed, is extremely rapid. Thus, like the other illustrations using nuclear DNA probes, it is suited to the screening of populations of plants gathered from the wild or created by plant breeders. In a recent survey of DNAs purified from maize lines gathered in Mexico, individuals were found lacking certain mitochondrial plasmid sequences (Kemble et al. 1983). These could have been detected much more rapidly using the techniques described here.

To maximise the value of the "squash-dot" method it will be necessary to isolate repeated sequences which can be shown to specify, or to be closely linked to, phenotypic characters important in genetics and plant breeding programmes. However, the assay method is not limited to detecting plant DNA sequences. It has already been shown to be useful for detecting alien nucleic acids, e.g. from viroids and viruses, in plant tissues (Owens and Diener 1981; Maule et al. 1983) and has been incorporated into the potato breeding programme at this Institute to detect viral infections (Baulcombe et al. 1984a, b).

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References

Baulcombe D, Flavell RB, Boulton R, Jellis G (1984a) The sensitivity and specificity of a rapid nucleic acid hybridisation method for the detection of potato-virus X. Plant Pathol 33:361–370

Baulcombe D, Flavell RB, Boulton R, Jellis G (1984b) The use of cloned hybridisation probes to detect viral infections in a potato breeding programme. Proc Phytochem Soc Symp (in press)

Bedbrook JR, Jones J, O'Dell M, Thompson RD, Flavell RB (1980) Molecular characterisation of telomeric heterochromatin in *Secale* species. Cell 19:545–560

Denhardt DT (1966) A membrane filter technique for the detection of complementary DNA. Biochem Biophys Res Commun 23:641–646

Duvick DN (1965) Cytoplasmic pollen sterility in corn. Adv Genet 13:2–56

Flavell RB (1982) Recognition and modification of crop plant genotypes using techniques of molecular biology. In: Vasil IK, Scowcroft WR, Frey K (eds) Plant improvement and somatic cell genetics. Academic Press, London New York, pp 277–291

Flavell RB, Kemble RJ, Gunn RE, Abbott A, Baulcombe D (1983) Application of molecular biology in plant breeding: the detection of genetic variation and viral pathogens. In: Nugent J (ed) Better crops for food. Pitman, London

Flavell RB, O'Dell M (1976) Ribosomal RNA genes on homoeologous chromosomes of groups 5 and 6 in hexaploid wheat. Heredity 37:377–385

Flavell RB, O'Dell M (1979) The genetic control of nucleolus formation in wheat. Chromosoma 71:135–152

Flavell RB, Smith DB (1974) The role of homoeologous group 1 chromosomes in the control of rRNA genes in wheat. Biochem Genet 12:271–279

Gahan PB, McClean J, Kalina M, Sharma W (1967) Freeze-sectioning of plant tissues. The technique and its use in plant histochemistry. J Exp Bot 18:151–159

Gale MD, Gregory RS (1977) A rapid method for early generation selection of dwarf genotypes in wheat. Euphytica 26:733–738

Gergen JP, Stern RH, Wensink PC (1979) Filter replicas and permanent collections of recombinant DNA plasmids. Nucleic Acids Res 7:2115–2136

Gerlach WL, Bedbrook JR (1979) Cloning and characterisation of ribosomal RNA genes from wheat and barley. Nucleic Acid Res 7:1869–1885

Grunstein M, Hogness DS (1975) Colony hybridisation: a method for the isolation of cloned DNAs that contain a specific gene. Proc Natl Acad Sci USA 7:3961–3965

Hutchinson J, Chapman V, Miller TE (1980) Chromosome pairing at meiosis in hybrids between *Aegilops* and *Secale* species; a study by in situ hybridisation using cloned DNA. Heredity 45:245–254

Jones JDG, Flavell RB (1982) The mapping of highly repeated DNA families and their relationship to C bands in chromosomes of *Secale cereale*. Chromosoma 86:595–612

Kemble RJ, Gunn RE, Flavell RB (1980) Classification of normal and male sterile cytoplasms in maize. 2. Electrophoretic analysis of DNA species in mitochondria. Genetics 95:451–458

Kemble RJ, Gunn RE, Flavell RB (1983) Mitochondrial DNA variation in races of maize indigenous to Mexico. Theor Appl Genet 65:129–144

Lonsdale DM, Thompson RD, Hodge TP (1981) The integrated forms of S1 and S2 DNA elements of male sterile mitochondrial DNA are flanked by a large repeated sequence. Nucleic Acids Res 9:3657–3669

Maniatis T, Jeffrey A, Kleid DG (1975) Nucleotide sequence of the rightward operator of phage λ. Proc Natl Acad Sci USA 72:1184–1188

Maule AJ, Hull R, Donson J (1983) The application of spot hybridisation to the detection of DNA and RNA viruses in plant tissues. J Virol Meth 6:215–225

Mettin D, Bluthner ND, Schlegel C (1973) Additional evidence on spontaneous 1B/1R wheat-rye substitutions and translocations. In: Sears ER (ed) Proc 4th Wheat Genet Symp. Mo Agric Exp Stn, Columbia Mo, USA, pp 179–184

Owens RA, Diener TO (1981) Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridisation. Science 213:670–672

Payne PI, Corfield KG, Holt LM, Blackman JA (1981) Correlations between the inheritance of certain high molecular weight subunits of glutenin and bread making quality in progenies of six crosses of bread wheats. J Sci Food Agric 32:57–60

Pring DR, Levings CS (1978) Heterogeneity of maize cytoplasmic genomes among male-sterile cytoplams. Genetics 89:121–136

Pring DR, Levings CS, Hu WWL, Timothy DH (1977) Unique DNA associated with mitochondria in the "S" type cytoplasm of male sterile maize. Proc Natl Acad Sci USA 74: 2904–2908

Rigby PWJ, Dieckmann M, Rodes C, Berg P (1977) Labelling DNA to high specific activity by nick translation with DNA polymerase. I. J Mol Biol 113:237–251

Sears ER (1954) The aneuploids of common wheat. M Agric Exp Stn Res Bull 572:1–59

Thompson RD, Kemble RJ, Flavell RB (1980) Variations in mitochondrial DNA organisation between normal and male sterile cytoplasms of maize. Nucleic Acids Res 8:1999–2008