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In situ hybridization to somatic metaphase chromosomes of potato

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Summary. An in situ hybridization procedure was developed for mitotic potato chromosomes by using a potato 24S rDNA probe. This repetitive sequence hybridized to the nucleolar organizer region (NOR) of chromosome 2 in 95%–100% of the metaphase plates. Another repetitive sequence (P5), isolated from the interdihaploid potato HH578, gave a "ladderpattern" in genomic Southern's of *Solanum tuberosum* and *Solanum phureja*, but not in those of *Solanum brevidens* and two Nicotiana species. This sequence hybridized predominantly on telomeric and centromeric regions of all chromosomes, although chromosomes 7, 8, 10 and 11 were not always labeled clearly.

Key words: In situ hybridization – Mitotic chromosomes – Potato – Repetitive sequences

Introduction

The technique of in situ hybridization of nucleic probes to metaphase chromosomes has been improved continuously since it was first established (Gall and Pardue 1969). In situ hybridization, using both radioactive and biotinylated probes to locate genes on meiotic and mitotic plant chromosomes, has mainly been achieved in cereal species like wheat (Hutchinson and Lonsdale 1982, Rayburn and Gill 1985) and in Allium (Jamieson et al. 1986). The chromosomes of these species are relatively large and the probes used in the studies were repetitive so that a signal of hybridization was easily located. In potato, an important crop species, only three genes have been localized so far by pachytene analysis (Yeh and Peloquin 1965, Ramanna and Wagenvoort 1976) and no known biochemical markers have been linked to a given chromosome. Knowledge about the organization of genes in the potato genome could be achieved by in situ hybridization. However, the mitotic chromosomes of potato are small, and for the use of pachytene chromosomes flowering plants are needed that are not always easy to obtain.

Recent results with the detection of single and low copy loci on the mitotic Crepis and Nicotiana chromosomes (Ambros et al. 1986a, b; Mouras et al. 1987) indicate that it is possible to detect sequences on relatively small chromosomes.

In the present paper we report the successful localization of two repetitive sequences: one of 2.3 kb in the NOR region and one of 1.4 kb in the telomeric and centromeric regions of all 12 potato chromosomes.

Materials and methods

Plant material

The monohaploid *Solanum tuberosum* H7322 (Mn 797322), the interdihaploid *Solanum tuberosum* HH578 and the dihaploid *Solanum phureja* SVP5 (PH 77-1445-2242) were used. The plants were grown in the greenhouse, 16 h photoperiod (3,000 Lux), 22 °C/18 °C day/night regime.

Plant DNA preparation and cloning

Plant DNA was isolated from etiolated HH578 potato plants by the method of Bedbrook (1981). Total EcoRI digests of potato DNA were used for cloning into plasmid pUC9 (Viera and Messing 1982).

Standard molecular techniques

Restricted potato DNA was separated on 0.7% agarose TBE gels (TBE is 89 m*M* Tris-borate, 89 m*M* Boric-acid, 2 m*M*

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EDTA), blotted onto Nitrocellulose filters and probed by nick-translated DNA according to Maniatis et al. (1982). Plant DNA containing pUC9 plasmids were screened according to standard colony hybridization methods (Maniatis et al. 1982) using nick-translated labelled HH578 nuclear potato DNA. Plasmid DNA was isolated according to Birnboim and Doly (1979). Inserts from plasmids were isolated by cutting the plasmids with the indicated restriction enzyme followed by agarose gel electrophoresis and the "Freeze-squeeze" method (Tautz and Renz 1983).

Preparation of slides

Root tips were pretreated in 0.2% α-Bromonaphtalene in tap water for 5 h at 4 °C. After fixation in cold absolute ethanol-glacial acetic acid mixture (3:1 v/v) for at least 2 h, the slides were made as described by Pijnacker et al. (1986). The enzymatic digestion lasted 1 h instead of 45 min. Slides with a sufficient number of metaphase plates were selected with phasecontrast microscopy and stored at − 80 °C until further use.

Synthesis of radioactive probes for in situ hybridization

Plasmid pJL4324, or the 2.3 kb EcoRI insert from this plasmid, containing the 24S nuclear ribosomal DNA (Landsmann and Uhrig 1985) and the large insert of plasmid P5 (this study) were used as probes. DNA probes were labelled by nick-translation using a BRL (Bethesda Research Laboratories, UK) nick-translation kit. DNA probes were labelled with ^{3}H dCTP (56 Ci/mmol) and ^{3}H TTP (118 Ci/mmol) in a 40 µl reaction mixture containing dGTP, dATP (25 µMol of each), 200 pg DNAse I, 2 units DNA polymerase I and 200–500 ng DNA in 100 m*M* Tris-HCl (pH 7.8). After 60 min incubation at 15 °C the reaction was stopped by adding EDTA and SDS. The mixture (final volume 200 µl) was passed through a Sephadex G-50 spun column to separate the nucleotides from the labelled DNA (Maniatis et al. 1982). Specific activities of $1 \times 10^8 - 5 \times 10^8$ dpm/µg DNA were obtained. A probe was denatured upon use at 90 °C for 10 min and immediately put onto ice.

In situ hybridization

Dry slides were treated with DNAse free RNAse A (24 µg/ml in 2 × SSC) for 1 h at 37 °C. The slides were washed briefly in 2 × SSC at room temperature, then for 30 min at 70 °C, again for 10 min at room temperature and then dehydrated in 70% ethanol. Chromosomes were denatured in 0.15 M NaOH in 70% ethanol at room temperature for 12 min (Landegent et al. 1984). The slides were then dehydrated in 70% ethanol (10 min), 100% ethanol (2 min) and air dried. Denatured chromosomes on a slide were covered with 40 µl of hybridization solution. The hybridization solution consisted of 50% formamide (Eastmann-Kodak, spectro grade), 100 µg/ml denatured salmon sperm DNA, 1 × Denhardts solution (Maniatis et al. 1982), 2 × SSC and 1–16 ng of labelled DNA (Pardue 1985). Cover slips, cleaned with absolute ethanol, were put over the hybridization solution. The slides were incubated in a humid chamber containing 50% formamide in 2 × SSC at 40 °C for 16–20 h. After incubation the cover slips were removed and the slides were washed once in 2 × SSC for 15 min at room temperature, once in 2 × SSC for 30 min at 37 °C and once in 2 × SSC at room temperature for 15 min.

The slides hybridized to ^{3}H-labelled probe were air dried and dipped in a 26 °C photographic emulsion (Ilford, Nuclear Research Emulsion in Gel Form, type L4) with a semi-automatic coating instrument (Vrensen 1970) and then stored at 4 °C for up to 6 weeks. The slides were ready for viewing after development and fixation. Giemsa staining for karyotyping was performed essentially as described before (Pijnacker and Ferwerda 1984).

Results

Isolation and characterization of repetitive probe P5

Nuclear DNA isolated from *S. tuberosum* HH578 and restricted with EcoRI was used in a ligation reaction with EcoRI cut plasmid pUC9. An alliquot of this mixture was transformed to *E. coli* JM83. Several hundred clones were obtained. By colony hybridization with labelled nuclear HH578 DNA, only those clones were selected that gave a strong signal. These colonies were the most likely to contain repetitive sequences, as these sequences will be present to a larger degree in the probe because of their repetition in the genome. One colony (P5) was chosen for the in situ hybridization experiments. Its selection was based upon: (1) the substantial stronger signal this positive clone gave; and (2) the so-called "ladderpattern" (Fig. 1, *lane 1*) it gave in genomic Southern's, which is characteristic for hybridization with a repetitive probe (Landsmann and Uhrig 1985, Cullis and Cleary 1986). Plasmid P5 contained two inserts, with sizes of 1.0 kb (P5S) and 1.4 kb (P5L). The inserts of plasmid P5 were not homologous to the 24S rDNA, nor did they show any hybridization with *Nicotiana tabacum, Nicotiana plumbaginifolia* or *S. brevidens*.

Denaturation of chromosomes in metaphase spreads

Initial experiments to obtain in situ hybridization following the denaturation procedures of Pardue (1985), Donlon (1986) or Rayburn and Gill (1985) failed. Therefore, we first estimated the degree of denaturation by staining the chromosomes with a drop of 0.01% acridine-orange in 1/15 M Sörensens phosphate buffer pH 6.9 and investigating them by fluorescence microscopy (filters

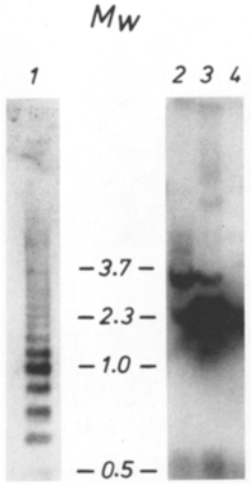

Fig. 1. Southern blot of EcoRI restricted potato DNA (3 µg/lane) hybridized with P5 (*lane 1*) and pJL 4324 (*lanes 2–4*). *Lane 1* and *4* HH578 DNA; *lane 2* H7322 DNA and *lane 3* SVP5 DNA. The numbers indicate molecular weights in kb

450–490 nm and 520 nm). Single stranded (denatured) DNA lights up orange, while double-stranded (not denatured) DNA is green. It appeared that a treatment of the slides in 70% formamide in 2 × SSC for 30 min at 70 °C was not sufficient to denature all the mitotic metaphase chromosomes. Moreover, the chromosomes were very swollen and not recognizable and, therefore, this procedure could not be used. Denaturation of the chromosomes in hybridization solution together with the probe did not produce fully denatured chromosomes either. By denaturing metaphase spreads in 0.15 M NaOH in 70% ethanol for 7–15 min all chromosomes were denatured and still in a good and recognizable shape. We always observed, however, a certain variation in the degree of denaturation depending on the quality and the genotype of the root tips.

Hybridization of chromosomes with an rDNA sequence

When using the 2.3 kb insert from pJL4324 as a probe (SA = specific activity 5×10^8 dpm/μg), hybridization sites occurred on the nucleolar organizer region of chromosome 2 (NOR; Fig. 2). The hybridization signals were consistent from metaphase to metaphase (95%–100% labelled) and from slide to slide. Non-specific or background hybridization was absent or occurred at a very low level. No hybridization signals were detected in control metaphase plates using unlabelled pJL4324, labelled plasmid without the insert or non-related plasmid pGV1 (Vosman et al. 1987). We observed, however, that when hybridizing the same labelled probe with H7322, HH578 (*S. tuberosum*) or SVP5 (*P. phureja*) chromosomes, the SVP5 nucleolar chromosomes contained 10–100 × more grains than chromosomes 2 of H7322 and HH578. To obtain a similar amount of silver grains, H7322 and HH578 slides had to undergo autoradiography for 6 weeks instead of 2 weeks. As can be seen in genomic Southern's (Fig. 1, *lanes 2–4*), 3 μg of SVP5 DNA gives a much stronger signal than 3 μg of H7322 or HH578 DNA. Thus, it seems that SVP5 contains more 24S rDNA copies per genome than the other two, which could account for the larger amount of silver grains in the metaphase plates of SVP5.

Another observation was made when using the 24s rDNA probe with a lower specific activity (10^7 dpm/μg) in combination with HH578 metaphase plates. In this case, the chromosome 2 with the largest satellite was labelled more often than the other chromosome 2. This difference was not noticed with higher specific activity probes ($>10^8$ dpm/μg).

Hybridization of S. phureja chromosomes with the P5L repetitive DNA sequence

When hybridizing P5L (SA 2×10^8 dpm/μg, 16 ng/slide) to SVP5 metaphase chromosomes, a signal could be obtained on nearly all chromosomes of all the metaphase plates after 6 weeks of autoradiography (Fig. 2d). Also in this case, no hybridization signal was found with unlabelled plasmid P5 or labelled plasmid pGV1 alone. Background hybridization occurred in a very low frequency and to a low degree. Labelling with P5L was predominantly found on telomeric regions, but also on centromeric regions. Maximally 22 of the 24 chromosomes of SVP5 became labelled. In one case the two unlabelled chromosomes were not homologous. Consequently, all chromosomes of the S. phureja genome can be labelled. The chromosomes that were not always la-

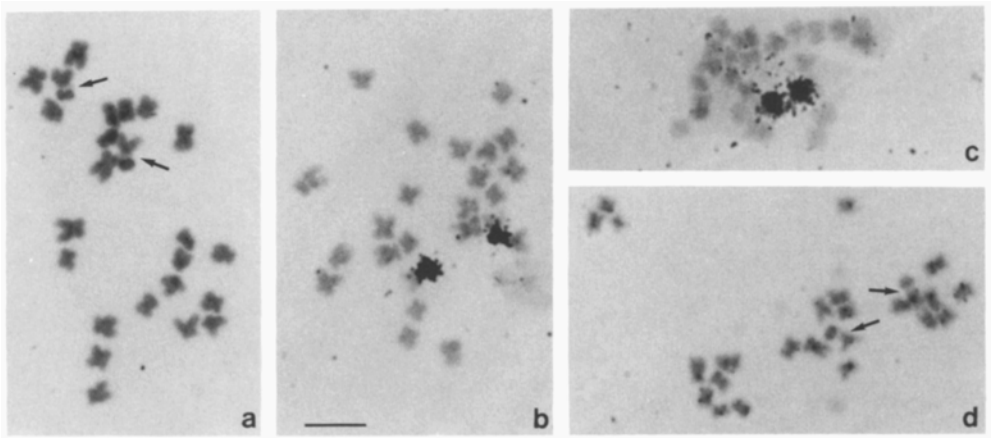

Fig. 2a–d. In situ hybridization of repetitive sequences with *Solanum* species. **a** *S. phureja* SVP5 metaphase chromosomes. **b** *S. phureja* SVP5 chromosomes hybridized with the 24S-rDNA, 5 ng probe 14 days autoradiography. **c** *S. tuberosum* HH578 chromosomes hybridized with the 24S-rDNA, 5 ng probe 6 weeks autroradiography. **d** *S. phureja* SVP5 chromosomes hybridized with P5L, 16 ng 6 weeks autoradiography. *Arrows* point to the nucleolar chromosomes in **b** and **c** covered by silver precipitate. *Bar* represents 5 μm

belled looked like chromosomes 7, 8, 10 and 11 of the S. tuberosum karyotype described by Pijnacker and Ferwerda (1984).

Discussion

The results presented in this paper show that it is possible to localize (repetitive) sequences on the relatively small metaphase chromosomes of potato. As pointed out by other investigators (for example Mouras et al. 1987), the success of localizing sequences in chromosomal DNA by in situ hybridization depends on several aspects of which the denaturation of the chromosomal DNA, the accessibility of the target DNA, the specific activity of the probe DNA and the preservation of DNA and chromatin during the entire procedure are the most important. Reckoning with these factors, we tried to get optimal results with the 24S-rDNA clone as a probe. By monitoring the degree of denaturation of the potato chromosomes with acridine orange staining we found that the normally employed denaturation procedures, like formamide or high temperature denaturation (Pardue 1985, Mouras et al. 1987), were not sufficient to denature all the chromosomes present in the metaphase plates of potato. Moreover, the chromosomes were not recognizable after denaturation by these two methods. We have found in the NaOH in ethanol denaturation a standard and reproducible method to denature mitotic potato chromosomes while maintaining them in a good recognizable shape.

With low specific activity probes longer exposure times are required for autoradiography, often giving rise to high background hybridization. Thus, high specific activity probes are necessary to get an optimal specific/nonspecific ratio. The source of the chromosomes is also very important as was observed in the case of HH578 chromosomes. In this particular case, the combination of a low specific activity of the 24S-rDNA probe and the source of chromosomes led to interesting results, one chromosome being labelled more often than the other. The chromosomes 2 of HH578 are not completely homologous, differing in their amount of C-banding of the NOR region (Pijnacker and Ferwerda 1987). This could well mean that one of the two chromosomes 2 has a smaller number of rDNA clusters and therefore a much fainter hybridization signal. When using probes with a higher specific activity, no difference could be seen between the hybridization signals of the chromosomes 2 of HH578. The results with this particular potato clone show that heterozygosity at a given locus can cause differences in the degree of hybridization. This may lead to difficulties when interpreting results of in situ hybridization experiments, particularly in the case of small target sites and/or low specific activity of the probe used.

The function of the DNA to which probe P5L hybridized is not yet clear. It is a repetitive sequence as can be seen in Southern hybridization. On the chromosomes, hybridization occurs predominantly in the telomeric and centromeric regions, suggesting that these regions must be rather identical in their sequences. These regions contain heterochromatin (Pijnacker and Ferwerda 1984, 1987), but this does not necessarely mean that P5L is a sequence of heterochromatic origin. Furthermore, it was found that P5L does not hybridize to Nicotiana species and to *Solanum brevidens*, an etuberosum member of the genus Solanum. The latter means that this repetitive sequence does not occur in *S. brevidens* and thus P5L might be specific for tuberous Solanum species. P5L is a suitable candidate to distinguish the tuberosum genotypes in the widely used somatic fusions between *S. tuberosum* and *S. brevidens* or between *Solanum tuberosum* and Nicotiana species (de Vries and Tempelaar 1987).

The results with the P5L probe show that even small (1.4 kb) probes can be used to efficiently localize sequences on the potato genome. Until now, the smallest fragment localized on mitotic chromosomes was the kanamycin sequence (2.4 kb) (Mouras et al. 1987). The next step will be to localize sequences that are less abundant to finally end up with the localization of single copy sequences on the mitotic potato chromosomes.

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