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DOI
10.1046/j.1365-313X.1996.9050767.x

Publication date
1996

Published in
Plant Journal

The Plant Journal (1996) **9**(5), 767–774

TECHNICAL ADVANCE

Detection of single-copy genes and chromosome rearrangements in *Petunia hybrida* by fluorescence *in situ* hybridization

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Summary

DNA sequences homologous to single-copy genes were labelled with biotinylated dUTP or digoxygenin-labelled dUTP and hybridized to chromosome spreads. The hybridization signals were visualized with fluorescent avidin- or antibody-conjugates. This method allowed the detection of DNA targets on metaphase chromosomes as small as 1.4 kb. The hybridization signals were identified as fluorescent spots on both sister chromatids. Using an 18S rDNA probe as marker to identify chromosomes II and III it was possible to assign single-copy genes to these chromosomes. In the line V30 the endogenous chalcone synthase gene (*chsA*) was mapped at the distal end of the short arm of chromosome 5. The cDNA probe for this single-copy gene was 1.4 kb. In contrast, in the lines Mitchell and V26 *chsA* was localized at the distal end of the long arm of chromosome 3, suggesting that a chromosomal rearrangement had taken place. In a transformed *Petunia uidA*, transgenes were detected using a 2.7 kb probe. One transgene was mapped on one of the homologues of chromosome II proximal to the ribosomal genes. This homologue could be distinguished from the other by having the ribosomal genes at the distal end of the long arm. Using multicolour fluorescence *in situ* hybridization it was shown that it is possible to detect the endogenous *chsA* genes and both transgenes simultaneously.

Received 30 January 1996; accepted 23 February 1996.
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Introduction

In plant cytogenetics, fluorescence *in situ* hybridization (FISH) has been used mainly to identify whole chromosomes (Schwarzacher *et al.*, 1989) and multi- or low-copy gene loci (Dong and Quick, 1995; Griffor *et al.*, 1991; Leitch and Heslop-Harrison, 1993; Leitch *et al.*, 1991; Montijn *et al.*, 1994). To detect single-copy plant genes as well as the transcripts coming from these genes, it is necessary to detect small targets, in the order of 1 to 2 kb. So far, such sensitive detections have been accomplished only on human chromosome preparations, where the sensitivity has even been increased to 0.25 kb (Richard *et al.*, 1994). The difficulty with plant cells is that they are surrounded by a wall that hampers penetration of the probe into the tissue to reach the target. For this reason Montijn *et al.* (1994) have optimized an embedding procedure of root tip cells to get access to the chromosomes with non-radioactive probes.

To locate a single-copy plant gene we have chosen chalcone synthase A (*chsA*) of *Petunia hybrida*. This gene is well characterized and in the inbred line V30 it has been assigned to chromosome V by RFLP analysis (Koes *et al.*, 1986, 1987). In addition, since *chsA* encodes the first enzyme of the anthocyanin pathway (Heller and Hahlbrock, 1980), it is a convenient reporter gene to study gene repression that is induced by transgenes (Napoli *et al.*, 1990; Van der Krol *et al.*, 1990). This phenomenon is known as co-suppression or sense-suppression. Some of the proposed models explaining this type of gene repression involve a stable or transient interaction between the transgenic loci and the endogenous loci (Flavell, 1994; Jorgensen, 1990; Matzke and Matzke, 1993). The localization of small DNA targets is therefore a prerequisite to study the effect of spatial gene position during gene expression. To be able to test the models for the repression of the *chsA* genes in *P. hybrida* we first attempted to detect *chsA* on metaphase chromosomes.

Here we describe the detection of *chsA* of three *P. hybrida* lines and show that targets as small as 1.4 kb can be visualized on metaphase chromosomes. In the course of this study we obtained some unexpected results regarding gene position and chromosome rearrangements that could not easily have been done without the FISH technique. The rearrangements include a putative translocation event

involving the chromosome that carried the *chsA* gene and an inversion of a chromosome that carried a transgene.

Results

Petunia *karyotype*

Young root tips contain cells at different stages of mitosis. To analyse the karyotype of *Petunia,* root tip cells from the line Mitchell were arrested in metaphase with 1-bromonaphthalene, fixed in ethanol/acetic acid (3:1) and squashed on slides. To identify the nucleolus organizing region (NOR) chromosomes the slides were hybridized to a biotinylated 18S rRNA gene probe (pSR1 2B2). The hybridization signals were detected with Texas Red-conjugated avidin, recorded with a cooled CCD camera and digitally processed to rearrange the chromosomes. The karyotype of the line Mitchell with $2n=14$ chromosomes is represented in pseudo-colours (Figure 1). The rRNA gene clusters, visualized in pink, are located at the distal end of the short arms of chromosomes II and III. These chromosomes were identified on the basis of their size and the subtelocentric position of the centromere. Chromosome II was further identified by the presence of satellites at the short arm. The rRNA genes in the lines V26 and V30 were also found on chromosomes II and III (not shown).

Detection and position of the chsA *gene in line V30*

We hybridized squashed root tips of the line V30 with a 1.4 kb *chsA* cDNA probe (pME1), that was labelled with biotin-16-dUTP. After hybridization, the probe was detected with FITC-conjugated avidin. Hybridization signals were considered positive when they appeared as fluorescent double spots on identical positions on the sister chromatids. The hybridization signals were recorded with the cooled CCD camera using an integration time of 5 sec. During processing the images were pseudo-coloured.

The hybridization signals of the *chsA* probe were found as yellow fluorescent spots at the short arm of two homologous submetacentric chromosomes (Figure 2). To facilitate the identification of the *Petunia* chromosomes, we reprobed the squash preparation with a digoxigenin-labelled 18S rRNA probe, which identifies chromosomes II and III. The results clearly show that in the line V30 the *chsA* genes are not located on NOR chromosomes (Figure 2). On the basis of chromosome size and the position of the centromere we concluded that *chsA* is located at the distal end of the short arm of either chromosome V or VI. These chromosomes are difficult to distinguish (cf. Figure 1). However, the physical location of *chsA* on chromosome V or VI in line V30 is in agreement with the genetic linkage data of Koes *et al.* (1987) who mapped *chsA* on chromosome V in V30.

In the lines Mitchell and V26, chsA *is located on chromosome III*

When chromosome preparations of the line Mitchell were hybridized with the *chsA* probe fluorescent double spots were found at the distal end of the long arm of two homologous subtelocentric chromosomes which can be recognized as chromosome III (Figure 3a). After reprobing the squash with the 18S rRNA probe and merging of the digital images the *chsA* and the 18S rRNA signals were found on opposite sites of chromosome III (Figure 3b). We therefore conclude that in the *Petunia* line Mitchell the *chsA* gene is located at the distal end of the long arm of NOR chromosome III, unlike the situation in line V30.

To find out whether the *chsA* position in Mitchell is an exception, we also tested line V26. Root tips from this line were hybridized with the *chsA* probe and the 18S rRNA probe. Both probes were labelled with biotin-16-dUTP and detected with Texas Red. The preparation was counterstained with DAPI (Figure 4a) and directly photographed from the fluorescence microscope. The 18S rRNA and *chsA* hybridization signals were found on opposite sites of chromosome III (Figure 4b) similar to the case of line Mitchell.

Double FISH detection of single-copy genes and chromosome rearrangement in a transgenic Petunia *line*

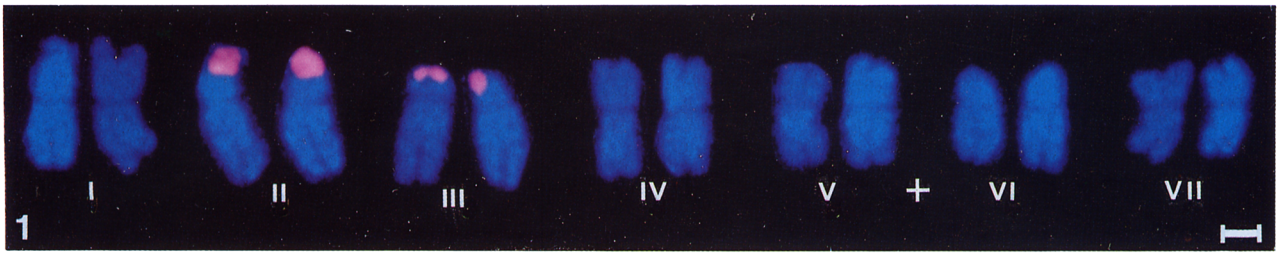
ChsA transgenes were located in the *Petunia* transformant S5055–14R, which is derived from PSE19–3 described by Van Blokland *et al.* (1994). The pSE19 T-DNA used to generate this transformant contains besides the *npt-II* selectable marker a chimaeric *chsA* gene consisting of the coding region of the β-glucuronidase gene (*uidA*) linked at its 3′ end to the 1.4 kb full-length *chsA* cDNA (Van Blokland *et al.*, 1994). This chimaeric gene is under the control of the CaMV-35S promoter. Southern blot analysis of genomic

Figure 1. Karyotype of *P. hybrida*, line Mitchell, after *in situ* hybridization with the 18S rRNA probe (pink spots, pseudo-coloured).
The image was recorded with a cooled CCD camera and digitized. Bar = 1 μm.

Figure 2. Fluorescence *in situ* hybridization of a metaphase spread from line V30 with *chsA* (yellow) and 18S rRNA (blue), recorded by a cooled CCD camera and pseudo-coloured.

Figure 3. Fluorescence *in situ* hybridization of metaphase spreads from line Mitchell recorded by a cooled CCD camera and pseudo-coloured. (a) after hybridization with *chsA* (yellow) and (b) after reprobing with 18S rRNA (pink).

Figure 4. Fluorescence *in situ* hybridization of a metaphase spread from line V26 with *chsA* and 18S rRNA recorded with a conventional photocamera.
(a) DAPI fluorescence image, (b) Texas Red signals of both probes. The *chsA* signal is indicated by a double arrowhead.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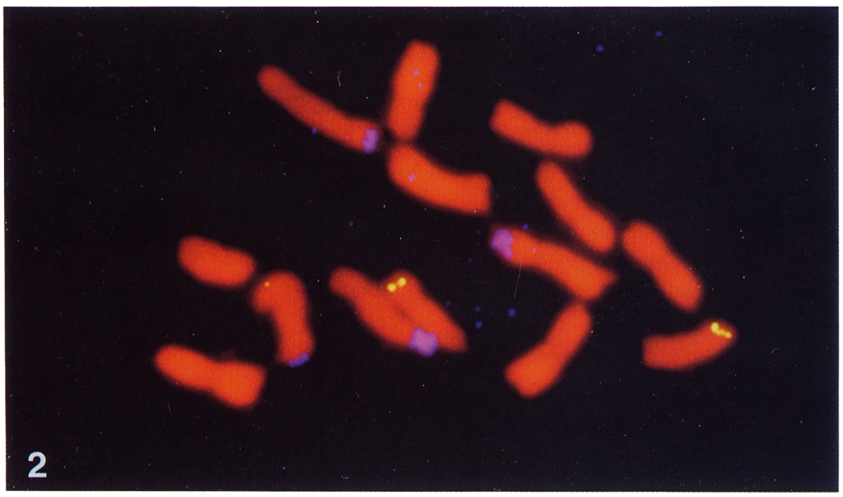
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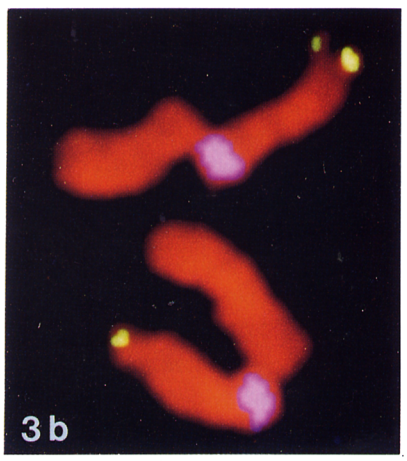
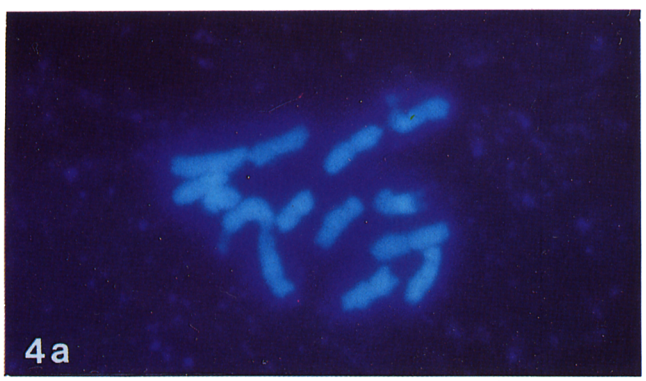
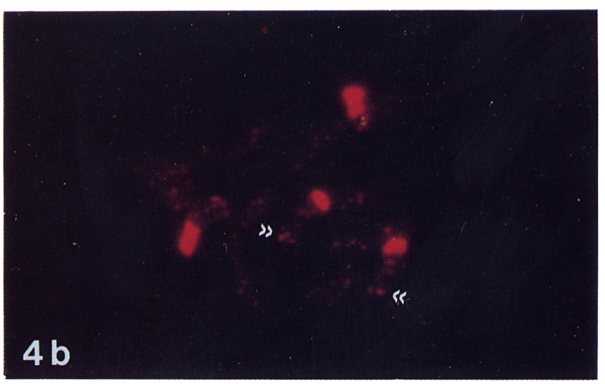
3a


3b


4a


4b

DNA showed that S5055–14R contains two T-DNA loci. One consists of a single, truncated T-DNA (S_t) missing the *npt-II* gene, whereas the other consists of two intact T-DNAs arranged as an inverted repeat around the right T-DNA border (IR_n; M. Stam, Kooter, unpublished results). Segregation analyses indicated that these two loci are on separate chromosomes (not shown). A first microscopic examination of the karyotype of the transformant revealed the presence of a dimorphic pair of satellite chromosomes (Figure 5a). One homologue has the secondary constriction at the short arm, which is normal in *Petunia* (Figure 1), whereas the other chromosome has the secondary constriction on the long arm. This suggests that a structural chromosomal mutation, probably a pericentric inversion, has taken place involving chromosome II. By *in situ* hybridization with the 18S rRNA probe the presence of ribosomal RNA genes on the long arm was confirmed (Figure 5b and c). After hybridizing with a digoxigenin-labelled CaMV-*uidA* probe one of the transgenes was found at the distal end of the long arm of the aberrant chromosome II proximal to the secondary constriction (Figure 5c). No hybridization signals were found on the normal homologue II. The consistent observation of a T-DNA hybridization signal on the long arm of the aberrant homologue II in different preparations proves the specificity of the FISH technique. The other T-DNA was mapped on chromosome IV, which could be identified by its size and the position of the centromere (not shown).

To simultaneously detect T-DNAs and endogenous *chsA* genes, chromosome squashes of the transformant R5055–14R were hybridised with probes for *chsA* and for the *uidA* genes of the T-DNAs. The *chsA* probe was labelled with biotin, whereas the CaMV-*uidA* probe consisted of a mixture of biotinylated and digoxigenin-labelled DNAs to be sure that the T-DNAs would give a two-colour fluorescence signal. The biotinylated probes were detected with Texas Red-conjugated avidin giving rise to red signals and the digoxigenin-labelled probes with fluorescein isothiocyanate (FITC)-conjugated anti-digoxigenin antibodies giving rise to green signals. The hybridization signals, recognized as double spots, were directly recorded from a conventional fluorescence microscope with a photocamera (Figure 6a–c). Green fluorescent double spots, detected with a FITC filter (495 nm), indicate the positions of the T-DNA targets (Figure 6a). Using a double band-pass filter (Omega) for FITC and Texas Red both *chsA* and the T-DNA targets were simultaneously detected as red and yellow double spots, respectively (Figure 6b). The yellow fluorescence is caused by the co-location of FITC-conjugated antibodies (green) and Texas Red-conjugated avidin (red) indicating that both the *uidA* probe and the *chsA* probe were hybridised to the same T-DNA target. The results demonstrate that it is possible to simultaneously detect and distinguish small targets at different loci, which is necessary to analyse the spatial positions in interphase nuclei of genes.

Discussion

This paper describes the application of FISH, to detect single-copy genes with 1.4 and 2.7 kb DNA probes. By comparison, targets of less than 1 kb have been detected with mammalian chromosomes (Fan *et al.*, 1990; Richard *et al.*, 1994). Despite the fact that the accessibility of plant chromosomes for probes may be obstructed by cell wall material and cell debris, the sensitivity approaches the results obtained with FISH on human chromosomes. Although the fluoresence signals could be detected by conventional fluorescence microscopy, digitizing the images by a CCD camera was of great value to amplify signals and to superimpose images from chromosome spreads after serial hybridizations with different probes. Regarding the intensity of the signals we expect that also in plants, targets smaller than 1 kb can be detected on metaphase chromosomes. The hybridizations with the *chsA* and T-DNA probes clearly show twin spots (Figure 2, 3a, 4b, 5d 6a and b), indicating both loci on the sister chromatids. In addition, the location of endogenous genes at identical positions on the homologous chromosomes further supports the specificity of the hybridization signal. Consequently, the analysis of chromosome spreads of only a few cells is sufficient to unequivocally map genes. This makes a tedious statistical evaluation of results obtained with radioactive probes unnecessary (Gustafson *et al.*, 1990; Huang *et al.*, 1988).

A major advantage of FISH above the radioactive methods is the possibility to hybridise several probes simultaneously or sequentially. This allows the identification of chromosomes by markers specific for each of the chromosomes. For example, the rRNA gene probes specifically recognize chromosomes II and III (Figure 1, 2, and 5). The principle has also been used by Leitch *et al.* (1991) and Heslop-Harrison *et al.* (1992) in plants to detect different repetitive sequences.

Detection of the endogenous chsA *gene*

As an example of an endogenous single-copy gene we have labelled the *chsA* gene. The *chsA* gene is a member of a multigene family (Koes *et al.*, 1987), but unlike other *chs* genes *chsA* does not seem to be clustered in tightly linked groups (Koes *et al.*, 1987). Of all *chs* genes, *chsJ* shows the highest identity (86%) with *chsA* with differences throughout the gene (Koes *et al.*, 1986). Unfortunately, the *chsJ* gene has not been mapped genetically. Any cross-hybridization between the *chsA* probe and the *chsJ* gene was avoided by the high wash stringency (0.1×SSC at 58°C). Nevertheless, some chromosome spreads showed

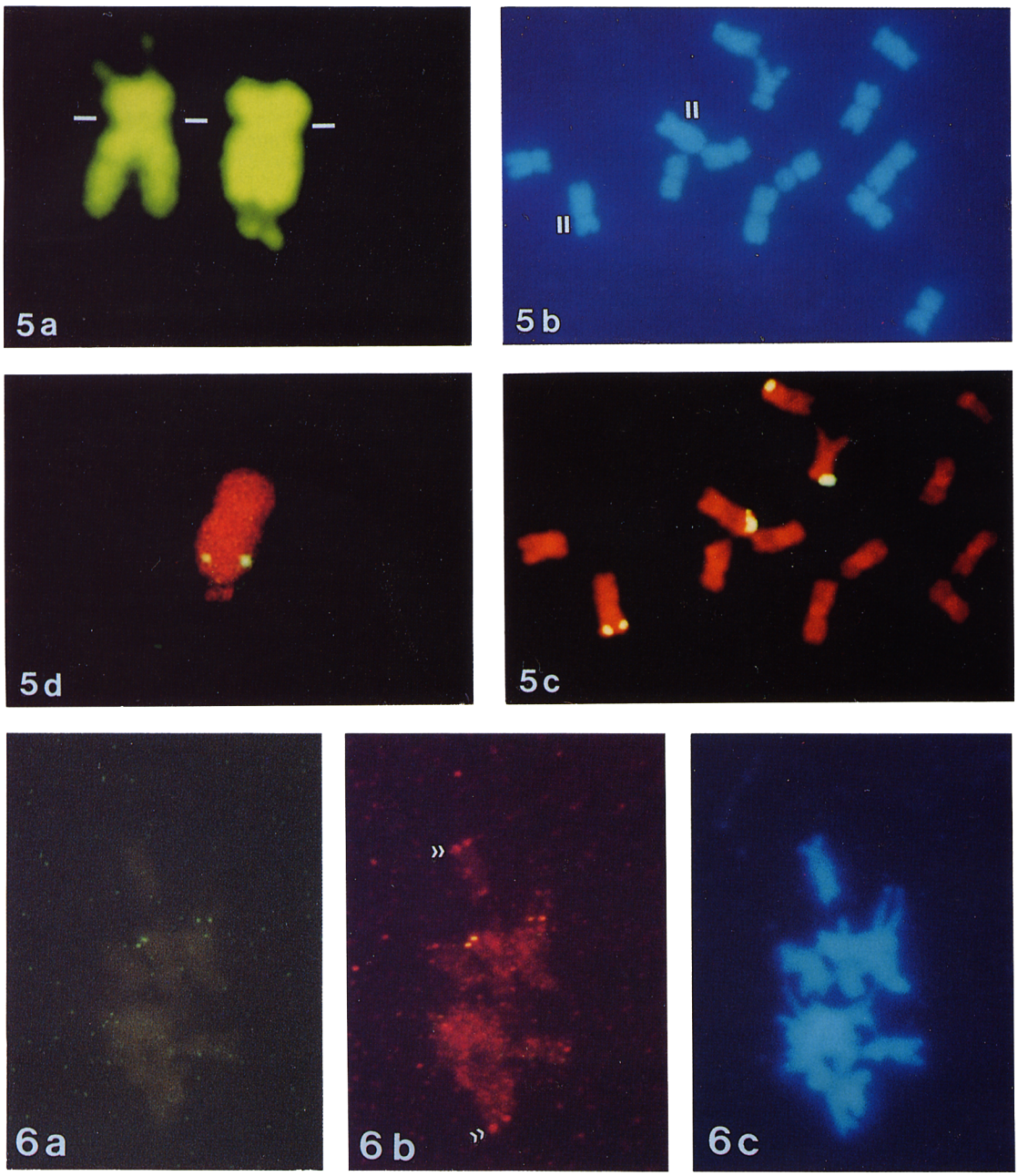


Figure 5. Fluorescent *in situ* hybridization to metaphase spread of S5055–14R. (a) A dimorphic pair of chromosome II stained with FITC using *in situ* labelling. Note the position of the satellites and the centromeres indicated between bars, (b) DAPI image and (c) pseudo-coloured hybridization signals after probing with 18S rRNA (yellow), (d) pseudo-coloured hybridization signal after probing with *uidA* (yellow).

Figure 6. Double-fluorescence *in situ* hybridization of a metaphase spread from S5055–14R with biotinylated *chsA* and with digoxigenin-labelled *uidA*, recorded with a conventional photo camera

(a) Fluorescein image showing the hybridization signals of digoxigenin-labelled *uidA* hybridized to the transgene loci.

(b) Simultaneous visualization of biotinylated (red) and digoxigenin-labelled probes (yellow). Co-location of both probes results in a yellow fluorescent signal. Red spots (double arrowheads) indicate the endogenous *chsA* gene. (c) DAPI-image.

cross-hybridization signals at the short arm of chromosome III (unpublished results). From this and from preliminary FISH experiments with a cDNA probe of the *chsJ* gene on V26 chromosomes we suggest that *chsJ* is located at the distal end of chromosome III, proximal to the NOR .

In line V30 we have mapped *chsA* at the distal end of the short arm of a chromosome that was morphologically identified as V or VI. This is in accordance with Koes *et al.* (1987), who found a weak but significant linkage with Hf2 by RFLP analysis. This phenotypic marker was assigned to the short arm of chromosome V (Cornu, 1984; Cornu *et al.*, 1980). However, the relative position of *chsA* on

chromosome V could not be determined, due to the lack of sufficient phenotypic markers.

In contrast to V30, in which *chsA* was mapped on chromosome V or VI, in the lines Mitchell and V26, *chsA* was found at the distal end of the long arm of chromosome III. The distal position on chromosome III has not been assigned for any of the *chs* genes (Koes *et al.*, 1986). Therefore, it appears that a chromosomal rearrangement has occurred in *Petunia* involving chromosomes III and V. This is further suggested by the distal positions of the gene on these chromosomes, assuming that a translocation event has taken place between these chromosomes (see below). Although a translocation of the ribosomal genes from chromosome III to chromosome V cannot be excluded, it is not likely, considering their position at the short arm in the lines used. Such a translocation would imply a highly complicated rearrangement. The structural chromosomal difference between V30 and the other *Petunia* lines, V26 and Mitchell, indicates that the genetic map of *Petunia*, which is based on seven linkage groups corresponding to each chromosome (Maizonnier and Moessner, 1979), has to be applied with care when using different lines.

Detection of transgenes

The FISH technique has facilitated the location of the T-DNAs in the transformant S5055–14R. One T-DNA was mapped at the long arm of chromosome II proximal to the ribosomal genes. This chromosome appeared to be the aberrant homologue of chromosome II, with an abnormal position of the NOR at the long arm instead of the short arm. Regarding the different positions of the satellites with respect to the centromere, the dimorphism is most likely due to a pericentric inversion of this chromosome in the transgenic line S5055–14R. The other T-DNA locus was mapped on chromosome IV. Without the FISH technique the aberrant chromosome II would not have been noticed, thereby complicating a genetic linkage analysis.

In conclusion, the FISH technique allowed us to physically map small DNA targets (1.4 and 2.7 kb) of endogenous genes and transgenes on plant metaphase chromosomes. The different locations of *chsA* in different *Petunia* lines, which was quite unexpected, indicates that spontaneous chromosomal rearrangements have taken place in the *Petunia* genome. The simultaneous detection of different single-copy DNA targets signifies an improvement in physical mapping of plant genes and is of great importance to address questions about the role of the spatial position of transgenes in establishing gene silencing.

Experimental procedures

Plant material

Root tips from the *Petunia hybrida* lines, V26, V30, Mitchell and from a transgenic S5055–14R were incubated in tap water saturated with 1-bromo-naphthalene for 2 h and subsequently fixed in fresh ethanol/acetic acid (3:1) for 3×1 h. The fixed samples were stored at –20°C until use.

Chromosome preparation

Fixed root tips were briefly rinsed in distilled water and macerated in an enzyme mix (0.1% (w/v) pectinase, 0.1% (w/v) cellulase and 0.1% (w/v) cytohelicase (Sigma) in 15 mM sodium acetate pH5.5) for 1–2 h at 37°C. After a short rinse in the same buffer the macerated root tips were softened in 50% acetic acid under a cover glass. Removal of cytoplasm was very important to obtain hybridization signals. Therefore, the softening step with acetic acid was critically followed. If necessary, the incubation was prolonged or the concentration of acetic acid was increased to 60%. After gently squashing, the slides were frozen in liquid nitrogen and the coverslip was removed with a razor blade. The specimens were dried overnight at 37°C and stored at 4°C.

The following plasmids were used: pSR1 2B2 contains a 1 kb *Eco*RI fragment of the 18S rRNA gene of soybean (Eckenrode *et al.*, 1985); pME1 contains a 1.4 kb *Bam*HI fragment of *chsA* of *Petunia*; pBI221 contains a 2.7 kb fragment including the CaMV–35S promoter and the β-glucuronidase gene (*uidA*) of *Escherichia coli* (Jefferson, 1987). The DNA probes were labelled with either biotin-16-dUTP (Boehringer Mannheim) or with digoxigenin-11-dUTP (Boehringer Mannheim) in a standard nick translation assay (Sambrook *et al.*, 1987). The labelled DNA probe was dissolved together with 50× excess salmon sperm DNA and 50× excess yeast tRNA in 50% deionized formamide (Fluka), 2×SSC, 50 mM sodium phosphate pH 7 to give a final concentration of 20 ng probe μl^{-1}.

In situ *hybridization*

In situ hybridization was performed as described by Wiegant *et al.* (1991) with some modifications (Montijn *et al.*, 1994). Slides with squashed root tips were rinsed in 2×SSC for 5 min followed by an incubation with RNase A (100 $\mu g\ ml^{-1}$, Sigma) in 2×SSC for 1 h at 37°C. Subsequently, they were rinsed in a Tris buffer (20 mM Tris–HCl pH 7.4, 2 mM $CaCl_2$) and incubated with proteinase K (0.1 $\mu g\ ml^{-1}$, Boehringer) in Tris buffer for 10 min at 37°C. After a short rinse in phosphate-buffered saline (PBS) (10 mM sodium phosphate pH 7.0, 143 mM NaCl) containing 50 mM $MgCl_2$ they were incubated with 1% (v/v) formaldehyde (Merck) in PBS for 10 min at room temperature, washed in PBS and then sequentially dehydrated in an ethanol series of 70%, 90% and 100%, 2 min each and air-dried. The DNA probes described above were hybridized in a single- or double-target hybridization assay. The hybridization mix consisted of 2–5 ng probe μl^{-1} for each target, 50× excess salmon sperm DNA, 50× excess yeast tRNA, 10% dextran sulphate, 50% formamide, 2×SSC, 50 mM sodium phosphate pH 7.0 in a final volume of 10–15 µl. The chromosome preparations with the hybridization mix were covered with a coverslip and incubated at 80°C for 2 min to denature the DNA. The slides were further incubated in a moist chamber for 16–20 h at 37°C. Posthybridization washes were performed in 50% formamide, 2×SSC pH 7.0 for 3×5 min at 42°C followed by a short rinse in 2×SSC at 42°C and finally in 0.1×SSC pH 7.0 for 3×5 min at 58°C.

In some cases two sequential hybridization assays were performed on the same chromosome squash. Slides were first hybridised as described above. After examination and recording the signals, the coverslips were removed and the slides were washed in PBS dehydrated in an ethanol series of 70%, 90% and

100%, 2 min each and air-dried. The hybridization with the second probe started with the denaturation step. During this step the probes of the first hybridization assay were removed. Alternatively, the probes and the chromosomes were separately denatured according to Lawrence *et al.* (1992). In this case after the formaldehyde fixation the slides were washed with 2×SSC and then incubated in 70% (v/v) formamide, 2×SSC pH 7.0 at 70°C for 2 min and immediately dehydrated in an ice-cold ethanol series (70%, 90% and 100%) and air-dried. Meanwhile, the probe was denatured for 5 min at 70°C.

Immunocytochemical detection

The multicolour detection of the probes was carried out according to Wiegant *et al.* (1991). All slides were pre-incubated in 4×SSC pH 7.0 (containing 5% non-fat dry milk; 4M) at 37°C for 30 min to prevent non-specific binding of the antibodies. All further incubation steps were performed at 37°C for 30 min followed by washing steps of 3×5 min at room temperature. The first detection step was carried out with Texas Red-conjugated avidin (5 μg ml^{-1}, Vector Laboratories) in 4M followed by washing for 5 min in 4T buffer (4×SSC, 0.05% (v/v) Tween-20) and 2×5 min in TNT (100 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20). The second detection step was performed with biotinylated goat-anti-avidin (5 μg ml^{-1}, Vector Laboratories) and mouse-anti-digoxigenin (0.2 μg ml^{-1}, Boehringer) in TNB (100 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% blocking reagent, Boehringer). The third detection step was carried out with Texas Red-avidin (see step 1) and FITC-conjugated rabbit-anti-mouse (1:1000, Sigma) in TNB. The fourth step was carried out with FITC-conjugated goat-anti-rabbit (1:1000, Sigma) in TNB. Subsequently, the slides were dehydrated in an ethanol series of 70%, 90% and 100%, 2 min each, air dried and mounted in Vectashield (Vector Laboratories) with 0.1 μg ml^{-1} diamidino-phenyl-indole (DAPI) as counterstain. Hybridization signals were directly photographed with a 640 ASA Scotch (3M) colour slide from a Leitz DM fluorescence microscope equipped with a double band-pass filter (Omega) for the simultaneous visualization of FITC (yellow) and Texas Red (red). Alternatively, hybridization signals were separately recorded with an Olympus HB2 fluorescence microscope equipped with a cooled CCD camera (Astromed), or with a confocal scanning laser microscope (Leica CSLM-fluovert). The digital images were processed on a Hewlett-Packard/Apollo 425 series workstation with Scilimage software (Ten Kate *et al.*, 1990).

In situ *labelling*

Slides with spread metaphase chromosomes were labelled *in situ* with the PRINS technique (Koch et al., 1989) using random primers. This technique improved the visualization of the satellites. Slides were pretreated with RNase, proteinase K and fixed in formaldehyde as described above for *in situ* hybridization. After a short wash in PBS (5 min) the slides were rinsed in 2×SSC for 10 min and denatured in 70% formamide, 2×SSC at 70°C for 2 min. The preparations were chilled in ice-cold 70% ethanol for 2 min and further dehydrated in 90% and 100% ethanol for 2 min each and air-dried. Ten microlitres of the labelling mixture were added to the slides. This mixture (10 μl) contained 50 mM Tris–HCl pH 7.8, 5 mM $MgCl_2$, 0.05 mg ml^{-1} bovine serum albumin, 0.05 mM dATP, 0.05 mM dGTP, 0.05 mM dCTP, 0.01 mM dTTP, 0.05 mM fluorescein-dUTP, 1 μl hexanucleotide mixture (62.5 A260 units ml^{-1} random hexanucleotides, Boehringer Mannheim), 0.2 U μl^{-1} Klenow enzyme (Boehringer Mannheim). The slides were covered with a 18×18 mm^2 coverslip and incubated in a moist chamber for 16–20 h at 37°C. The preparations were washed in 2×SSC for 3×15 min, dehydrated in an ethanol series (70%, 90%, 100%), air-dried and mounted in Vectashield.

Acknowledgements

This work was supported by a grant of the Netherlands' Organization for Scientific Research (NWO) to the graduate school BioCentrum Amsterdam (P.F.F.), by a grant of the Netherlands' Technology Foundation (STW) to M.S., and by a research fellowship to J.M.K. from the Royal Netherlands Academy of Arts and Sciences (KNAW). We thank Jos Mol for critically reading the manuscript.

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