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Localization of T-DNA Insertions in Petunia by Fluorescence in Situ Hybridization: Physical Evidence for Suppression of Recombination

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Using fluorescence in situ hybridization (FISH) with metaphase preparations, we localized a 4-kb single-copy T-DNA sequence in a group of petunia transformants. The selected T-DNAs previously had been shown to be linked to the phenotypic marker *Fl* on chromosome II. Linkage analysis had revealed that recombination around the *Fl* locus is suppressed in a wide cross relative to an inbred recombination assay. The localization of six *Fl*-linked T-DNAs and the *Fl* locus itself, using FISH, revealed a number of aspects of recombination in petunia: (1) the central region of chromosome II showed at least a 10-fold suppression of recombination in wide crosses relative to the distal region; (2) recombination in wide hybrids over two-thirds of the chromosome was extremely low; and (3) recombination between completely homologous chromosomes in an inbred cross also was suppressed in the central region. In addition, the T-DNAs were not evenly distributed along the chromosome, suggesting a possible preference for a distal position for T-DNA integration. Implications for such a preference are discussed.

INTRODUCTION

The ease of plant transformation using Agrobacterium has led to a large resource of transformed plants for use in both fundamental and applied research. After infection with Agrobacterium, the T-DNA is directed to the nucleus by means of the VirD2 protein, which has several nuclear-localizing signals (Koukolikova-Nicola and Hohn, 1993). A high proportion of T-DNA inserts have been found in transcriptionally active chromatin (Ingelbrecht et al., 1991). T-DNA integration, as such, occurs by illegitimate recombination, and there is no preference for specific DNA sequences (Mayerhofer et al., 1991). Therefore, one might anticipate that T-DNA integration occurs essentially at random with respect to the euchromatic genome. Genetic localization of T-DNA markers in tomato (Chyi et al., 1986; Thomas et al., 1994) and petunia (Wallroth et al., 1986; Robbins et al., 1995) seems to support the idea of random T-DNA integration in the genome.

In an extensive study, 78 independent T-DNA loci were mapped with respect to marker genes for five of the seven petunia chromosomes (Robbins et al., 1995). The distribution of 49 mapped T-DNAs between the five marked chromosomes was approximately consistent with their physical size. A detailed analysis for a marker on chromosomes I (*Hf1*) and II (*Fl*) revealed that one-third of the T-DNAs were significantly linked to either marker. Moreover, 40% of these linked T-DNAs mapped within 1 centimorgan (cM) of the chromosome-specific marker. This remarkable outcome could result from T-DNA integration hot spots at both marker loci. An alternative interpretation is that a general suppression of recombination occurs around these marker loci in the F_1 hybrids used for mapping. Evidence in support of the latter hypothesis is provided by a recombination assay between transgenes in an inbred background (Robbins et al., 1995).

To distinguish between the above interpretations, we have taken a direct physical approach to map selected T-DNAs on petunia chromosomes. We combined the availability of genetically defined transformants of an earlier study (Robbins et al., 1995) with the technique to detect single-copy genes with fluorescence in situ hybridization (FISH) (Fransz et al., 1996). We report the localization of six closely linked T-DNA loci that span almost the entire length of chromosome II, thus providing no evidence in support of an integration hot spot. The comparison of the cytological data with the genetic data on wide crosses between petunia inbred lines revealed that a dramatic suppression of recombination occurs in the central region of chromosome II. Moreover, the cytological position of the six T-DNAs suggests possible positions of another eight

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genetically localized T-DNAs on chromosome II, indicating a preference for distal integration.

RESULTS

T-DNA Copy Number

To demonstrate the sensitivity of the FISH method for the detection of small DNA targets, we have determined the actual size of the targets in two of the petunia transformants. DNA gel blot analysis to determine the copy number of integrated T-DNA sequences is presented in Figure 1. Total genomic DNA of the two homozygous lines Ac9 and Ac158 was digested with BamHI, BclI, and NcoI. The digested DNA was hybridized with an *Activator* (*Ac*) probe that recognized the *Ac* transposon sequence in the JJ2853 T-DNA.

The BamHI restriction site occurs twice in JJ2853 T-DNA (Jones et al., 1989), and the *Ac* probe recognizes a single border fragment in the genomic DNA of both lines (Figure 1B, lanes 1 and 2). BclI (Figure 1B, lanes 3 and 4) cuts twice in the JJ2853 T-DNA, once inside and once outside the *Ac*-probed sequence. One fragment of ~5 kb is expected, and a second fragment is of unknown size. However, line Ac9 only produces one band, suggesting the loss of one BclI restriction site in the integrated T-DNA. Finally, NcoI has several restriction sites in the JJ2853 T-DNA and should produce an *Ac*-hybridizing fragment of 7.5 kb if a single copy of the T-DNA is present in the genome. The line Ac158 produces the 7.5-kb fragment (Figure 1B, lane 6), but the line Ac9 produces a high–molecular weight fragment (Figure 1B, lane 5), implying the loss of one of the NcoI sites. The hybridizing pattern of Ac9 suggests that, although the original structure of the JJ2853 T-DNA sequence is not integrated intact in the genome, the T-DNA is single copy.

FISH of the *Fl* Gene

We have used the FISH technique as shown in Figure 2 to map the physical position of T-DNA sequences (see below) as well as the flavonol synthase gene (Holton et al., 1993). Flavonol synthase, which is the genetic marker *Fl* on chromosome II, could be localized with a cDNA clone of 1187 bp. The fluorescently labeled chromosomes were digitally recorded with a cooled CCD camera mounted on a fluorescence microscope. The digital images obtained, as shown in Figures 3 and 4, enabled the application of computerized measurement of the spot position on the chromosomes (see Methods). As shown in Table 1 and Figure 5, the physical position of *Fl* could be localized on the long arm of chromosome II close to the centromere, both at a mean relative position of 0.67.

FISH of T-DNA

The *Ac* lines that were genetically characterized by linkage analysis in most detail were those showing T-DNA linkage to

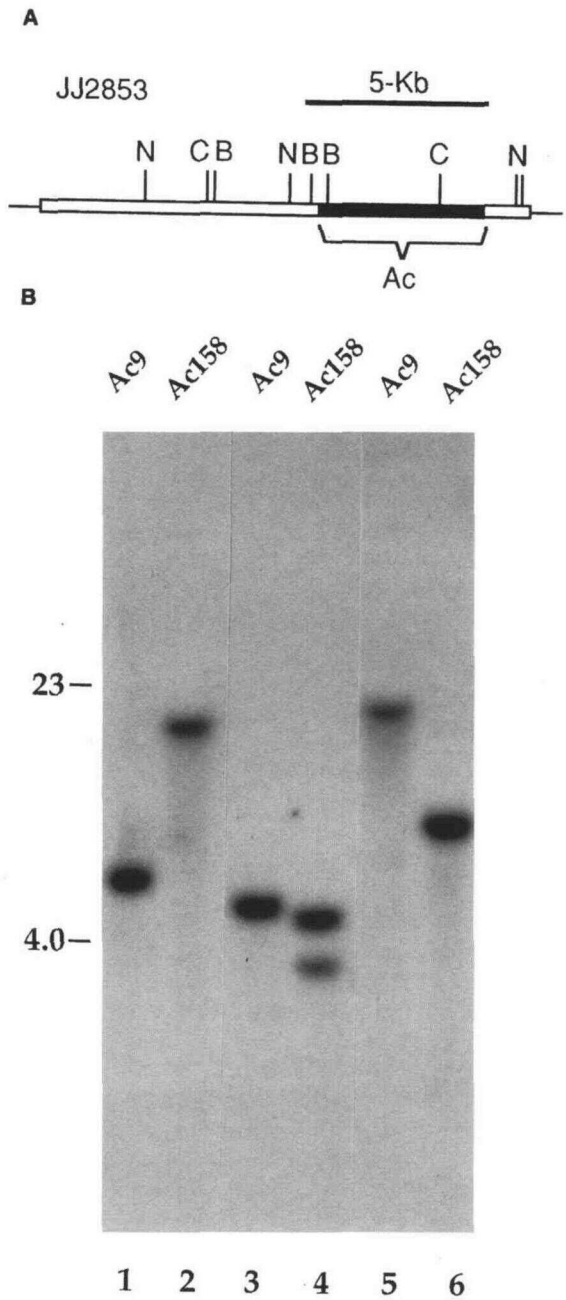

Figure 1. DNA Gel Blot Analysis of T-DNA Copy Number in *Ac* Transformants.

(A) T-DNA region of the binary vector plasmid JJ2853 with which the *Ac* lines were transformed. The black bar represents the *Ac* transposon. B, BamHI; C, BclI; N, NcoI.

(B) Autoradiography of a gel blot of 10 μg of total genomic petunia DNA of Ac9 and Ac158 digested with BamHI, BclI, and NcoI. The blot was hybridized with an *Ac* probe (see Methods). Lane 1 contains Ac9 cut with BamHI; lane 2, Ac158 cut with BamHI; lane 3, Ac9 cut with BclI; lane 4, Ac158 cut with BclI; lane 5, Ac9 cut with NcoI; and lane 6, Ac158 cut with NcoI. The unique band produced with BamHI shows that both transgenic lines had single-copy insertions. Numbers at left are in kilobases.

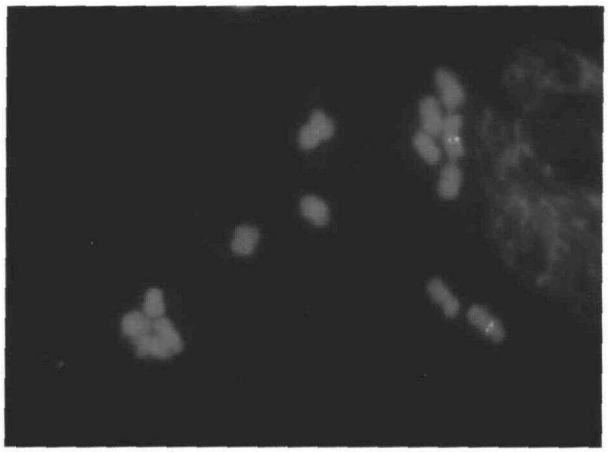

Figure 2. FISH of T-DNA Insertion on Metaphase Chromosome Spreads of the Petunia Transformant Ac21.

Direct microscopy from Ac21 labeled with an *Ac* probe (yellow spots). The *Ac* probe hybridized with T-DNA; total DNA was counterstained with propidium iodide (red).

Hf1 and *Fl* (Robbins et al., 1995). Localizing T-DNAs of these transgenic lines would provide the data to compare genetical and physical position on chromosomes I and II. The group of chromosome II–inserted T-DNAs was most suited for this purpose because the subtelomeric chromosome II allowed an unequivocal discrimination between the long and short arms. This discrimination between the two arms was not possible for the metacentric chromosome I. The measured positions of the T-DNAs of six *Ac* lines are shown in Table 1 and outlined in Figure 5. The genetic data of chromosome II–inserted *Ac* lines of both the wide crosses and the inbred crosses also are depicted in Figure 5. The order of the markers on the genetic map has been derived from the physical map constructed with our FISH data.

The T-DNAs of transgenic lines Ac145, Ac118, Ac21, and Ac80 were tightly linked within 1 cM to the phenotypic marker *Fl* (Table 1). On the physical map, they are dispersed (Figure 5). For example, the Ac145 and Ac80 T-DNAs are localized at relative positions 1.00 and 0.33, respectively. The recombination distance to *Fl* for the lines Ac145 and Ac80 as established in the mapping crosses assay is only 0.9 and 0.6 cM, respectively. The genetic distance between these two transgenes should be 1.5 cM. The lines Ac9 and Ac158, at 2.2 and 11.6 cM, respectively, from the marker gene *Fl* close to the centromere, proved to be physically localized at the distal part of the long arm.

Of the six *Ac* lines examined by FISH, four were homozygous and thus show two double spots at identical positions on both homologous chromosomes (see, for example, the visualized T-DNA in line Ac21 in Figure 2). Although no homozygous line of Ac80 and Ac118 was available for this study, we could discern the positions of Ac80 and Ac118 via the F_1 hybrids, as is schematically depicted in Figure 6. In the F_1 hybrids, one T-DNA has been derived from one parental *Ac* line, and the other T-DNA has been derived from the other parental *Ac* line. The physical position of the T-DNA of homozygous Ac9 was established by FISH. In the F_1 hybrid Ac9/Ac80, one T-DNA had the relative position that agreed with the Ac9 T-DNA position; therefore, the other localized T-DNA in the same metaphase spread should have been derived from the Ac80 parent. By comparing the fluorescence signals of F_1 hybrids Ac9/Ac80, Ac9/Ac118, Ac80/Ac118, and the homozygous Ac9, the T-DNA positions for Ac80 and Ac118 could be identified (Figure 6).

DISCUSSION

Detection of Small Targets with FISH

T-DNA duplications frequently occur during integration of T-DNA in leaf disc inoculation in an Agrobacterium culture (Deroles and Gardner, 1988; Grevelding et al., 1993). Thus, the target for detection of T-DNA would possibly be much longer than the supposed 4.6 kb (one copy of the *Ac* transposon) from a single-copy T-DNA. Therefore, we checked the target size of the transgenes integrated in the *Ac* lines. We established by DNA gel blot analysis that the Ac9 and Ac158 lines had single-copy T-DNA integrations (Figure 1). Each chromosome bearing a single-copy target T-DNA in metaphase spreads showed two fluorescence spots. Clearly, a single-copy 4.6-kb *Ac* transposon was an effective target for hybridization. The

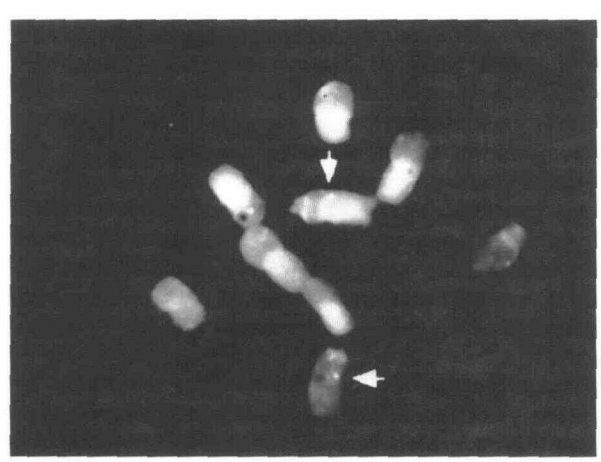

Figure 3. FISH of the Flavonol Synthase Gene of the Petunia Inbred Line V26.

Shown are V26 chromosomes labeled with a cDNA clone for the flavonol synthase gene. This CCD recorded image shows the labeled cDNA probe as a bright white double spot (arrows) visible on autofluorescent petunia chromosomes II.

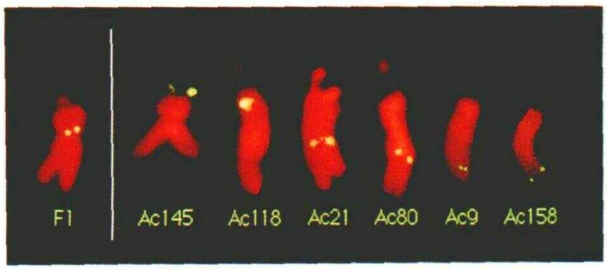

Figure 4. Selected Chromosomes II of Petunia *Ac* Transformants with the *Fl* Gene and T-DNA Visualized by FISH.

Shown are digital images of chromosomes of the *Ac* lines exhibiting the fluorescence-labeled double spot signal of the T-DNA as recorded with a CCD camera. The FITC fluorescence image and the 4',6-diamidino-2-phenylindole (DAPI) fluorescence image are combined in a pseudocolor image, selected, and aligned with Adobe Photoshop (Mountain View, CA). FITC is visualized in green, and DAPI staining is red for clarity.

smaller target of the *Fl* gene also was detected, but the double spot signal had a weaker fluorescence. The centromere position and the *Fl* position as measured by FISH could not be distinguished. *Fl* has been positioned cytogenetically on the long arm of chromosome II (Maizonnier et al., 1986). Thus, *Fl* is located on the long arm close to the centromere.

Suppression of Recombination around the *Fl* Locus

Genetic mapping of T-DNAs based on wide crosses revealed an apparent clustering of T-DNA loci on chromosomes I and II. However, when a selection of T-DNA loci closely linked to either *Hf1* or *Fl* were mapped relative to each other in an inbred genetic background, recombination rates were much higher than anticipated from the wide crosses (Robbins et al., 1995). This suggested that the apparent clustering of T-DNAs around the marker genes in the original map might have resulted not from any preference for T-DNA integration but rather from a suppression of recombination in the wide crosses. The physical mapping of T-DNAs on chromosome II confirms this interpretation. Most dramatically, the interval between Ac118 and Ac80 should not exceed 0.9 cM, according to the genetic mapping to *Fl*, yet accounts for 63% of the chromosome length.

Distally located T-DNAs of transformants Ac9 (2.2 cM) and Ac158 (11.6 cM) are at 0.09 and 0.05 relative positions, respectively (Figure 5). More centrally positioned T-DNAs are those in transformants Ac21 (0 cM) and Ac80 (0.6 cM) (Figure 4 and Table 1). For recombination in the distal segment, this amounted to 9.6 cM for 4% of the chromosome length (2.4% recombination for 1% chromosome length; see Table 2). For the central segment between Ac21 and Ac80, this recombination was only 0.6 cM for 13% of the chromosome length (0.05% recombination for 1% chromosome length), which is similar to the other central segment between Ac80 and Ac9, showing 1.6 cM for 24% of the chromosome length (0.06% recombination for 1% chromosome length; see Table 2). Considering these data, the central segment has a 42-fold suppression of recombination relative to the distal segment (Table 2). A similar difference in recombination level between centromeric and telomeric regions also was established in Drosophila (Roberts, 1965).

Possible Reasons for Suppression of Recombination in Petunia

An explanation for the observed difference in recombination frequency in different crosses might be the presence of recombination-enhancing genes. These could be either dominant as in the case of the *Rm1* gene (recombination modulator) in petunia (Robert et al., 1991) or recessive as in the case of *rec1* in *Caenorhabditis elegans* (Rose and Baillie, 1979). Because there are presumably multiple genes that modulate recombination, inbreeding favors the expression of recessive genes, whereas crossing between different lines with different genotypes inhibits recessive alleles to function upon recombination. However, the presence or absence of *Rm1* is insufficient to explain the magnitude of suppression of recombination in the wide cross versus the recombination in the inbred cross.

An alternative explanation for the observed suppression of recombination is that considerable structural polymorphism existed between the chromosomes of the wild species that were hybridized to create petunia (Sink, 1984). Such polymorphism is an integral feature of speciation in Drosophila and also has been documented in the plant genus *Crepis* (White, 1978). Evidence from the analysis of random amplified DNA polymorphism in petunia and its presumed ancestors indicates that chromosomal blocks of markers have indeed remained intact (Peltier et al., 1994). In addition, certain genes may occur on different chromosomes in different lines: the chalcone synthase A gene family in petunia (P.F. Fransz, unpublished results) or the 5S rRNA genes in petunia (M.B. Montijn and R. ten

Table 1. Relative Physical Distance of the T-DNA Insertions in Various Transgenic *Ac* Transformants

Ac Transformant	Map Distance (cM from *Fl*)[a]	Relative Physical Distance[b]	No.[c]
Ac145	0.9	1.00	10
Ac118	0.3	0.96 ± 0.04	9
Ac21	0.0	0.46 ± 0.06	33
Ac80	0.6	0.34 ± 0.06	11
Ac9	2.2	0.09 ± 0.04	7
Ac158	11.6	0.05 ± 0.05	6

[a] Data from Robbins et al. (1995).
[b] Total chromosome length is 1.00.
[c] No., number of chromosomes analyzed.

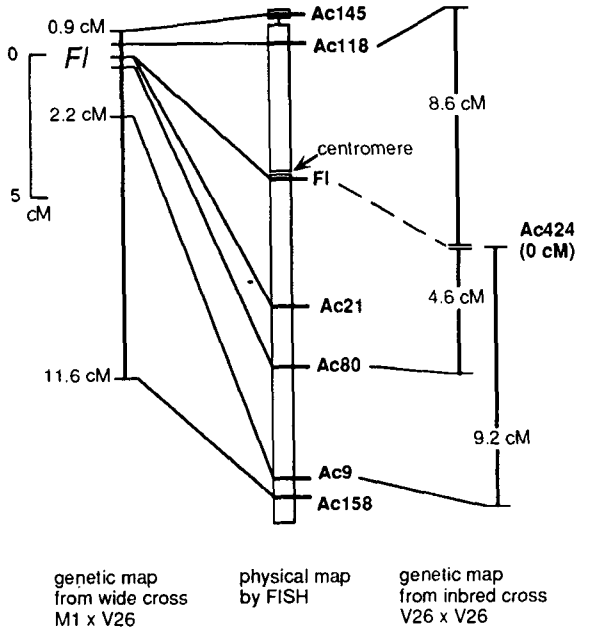

Figure 5. Schematic Representation of the Physical Data versus the Genetic Data of T-DNA Position on Chromosome II.

Three maps are shown. At left is the genetic map from M1 × V26 wide cross with the centimorgan distance of the T-DNA markers from phenotypic marker *Fl*. In the center is the physical map constructed by FISH. Bars in the chromosome indicate physical position. At right is the inbred recombination map showing the established centimorgan distances between transgenes.

Hoopen, unpublished results) by using FISH. The resulting discontinuities between chromosomes of different lines could inhibit recombination dramatically. Any recombination events that do occur between ectopic sites are likely to produce inviable gametes that would not be transmitted.

Preferences for T-DNA Integration

The direct physical mapping of transgenes rules out a preference for integration around the marker gene *Fl*. However, our results suggest other preferential sites for T-DNA integration in the genome of petunia. Fourteen of the hybrids used for linkage analysis of 84 kanamycin-resistant transformants showed linkage to *Fl* (Robbins et al., 1995). Of these chromosome II–inserted transgenes, six were genetically more distant than Ac9 (at 2.2 cM), which is physically located at the relative position 0.09. Thus, it appears that 50% of the investigated transgenes were integrated in the distal 10% of the long arm of chromosome II.

Results from other groups also indicate a distal preference for integration; seven out of 11 T-DNA inserts were detected in the distal region in a group of transformants of petunia (Wang et al., 1995). Also, three of five *C. capillaris* transformants were telomeric (Ambros et al., 1986), and a genetic analysis of 21 tomato transformants placed most of the T-DNAs in the distal region (R. Weide, personal communication). All these data point to the distal regions of chromosomes in the plant genome as preferential sites for integration of T-DNA. Interestingly, the telomeric regions have been reported to be located in the periphery of pea nuclei (Rawlins and Shaw, 1989).

A preference for a high proportion of T-DNAs integrated in distal regions could have a number of causes. According to Dietrich (Dietrich et al., 1981), the heterochromatin region is mainly located around the centromere in petunia. Insertion in the heterochromatin of the centromeric region could result in poor transgene expression, and thus, loss of lines with such a T-DNA insert would result in a biased population of predominantly distal-inserted T-DNAs. Second, the proximal region could remain more condensed during the different stages of the cell cycle at which T-DNA integration occurs, creating a physical barrier for T-DNAs to insert at these regions of the chromosome. It has been reported that T-DNA inserts are relatively abundant in transcriptionally active chromatin because 30% of T-DNA inserts without promoter are expressed, suggesting intragenic insertion (Ingelbrecht et al., 1991). Finally, a positive reason for a distal integration site could be that the distal DNA is readily accessible for integration by its position near the place of entry of the T-DNA in the plant nucleus. Additional research will focus on the three-dimensional localization of T-DNA inserts in intact plant nuclei.

METHODS

Petunia hybrida Lines

Four inbred petunia transformants were studied: Ac9, Ac21, Ac145, and Ac158. Three hybrid transgenic lines used were Ac9/Ac80, Ac9/Ac118,

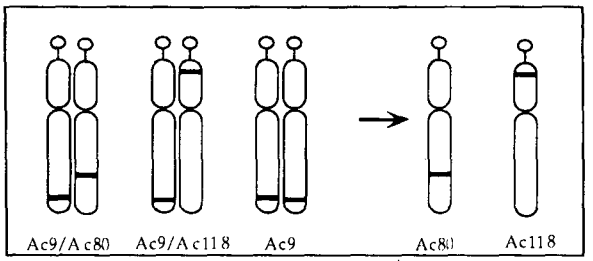

Figure 6. Chromosome II of Hybrids: Identification of T-DNA of Ac80 and Ac118.

Shown is a schematic representation of the chromosome II homologs with indicated T-DNA position of hybrids Ac9/Ac80, Ac9/Ac118, and the homozygous Ac9. The T-DNA position of Ac80 and of Ac118 can be determined by comparison of the positions in these different hybrids.

Table 2. Genetic and Physical Distances

Chromosome Segment	Genetic Distance: Inbred Crosses (cM)	Genetic Distance: Wide Crosses (cM)	Physical Distance[a]	cM/1% L: Inbred Crosses[b]	cM/1%: Wide Crosses[b]
Ac118 to Ac80	13.2	0.9	0.63	0.2	0.02
Ac80 to Ac9	4.6	1.6	0.24	0.2	0.06
Ac9 to Ac158	ND[c]	9.6	0.04	ND	2.4

[a] Total chromosome length is 1.00.
[b] Relative length. Total chromosome length is 100%.
[c] ND, not determined.

and Ac80/Ac118 (Robbins et al., 1995). The T-DNA in all cases is the chimeric construct JJ2853 (Jones et al., 1989) shown in Figure 1A and contains a 4.6-kb *Activator* (*Ac*) transposon sequence from *Zea mays*, inserted in the promoter region of a streptomycin resistance gene (*SPT*). In addition, it contains the 1.5-kb kanamycin resistance gene *NPT II*, encoding neomycin phosphotransferase II, and *Agrobacterium tumefaciens* Ti plasmid sequences.

Plant Growth and Harvesting

Plants were grown in a greenhouse for 6 to 8 weeks. Metaphase accumulation was performed by submerging freshly harvested root tips, ~3 cm in length, in water that contained a saturating amount of α-bromo-naphthalene (10 μL/100 mL of water) for 1.5 to 2 hr at room temperature. Root tips were fixed in ice-cold absolute ethanol:acetic acid (3:1 [v/v]) and replenished three times. Fixed root tips were stored at −20°C until further treatment.

Plant DNA Isolation and Gel Blot Analysis

Total genomic plant DNA was isolated by a modified cetyltrimethylammonium bromide extraction method (T.P. Robbins and R.M. Harbord, unpublished data) and digested with restriction endonucleases (BamHI, NcoI, and BclI), electrophoresed, and blotted onto Duralon-uv nylon membrane (Stratagene). DNA was covalently bound to the nylon membrane by irradiation with UV and hybridized according to the manufacturer's instructions in an aqueous formamide-free buffer at 65°C. Membranes were washed at 65°C in 0.2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% (v/v) SDS.

Chromosome Preparation

Fixed root tips were washed in distilled water followed by a rinse in 15 mM NaAc, pH 5. Root tips were digested in 1% (w/v) enzyme mix pectolyase (Sigma P9932), cytohelicase (Sigma C8274), and cellulase (Sigma C2415), 1% (w/v) each, in 15 mM NaAc for 1 to 2 hr at 37°C in a moist chamber. They were then rinsed in 15 mM NaAc, pH 5. Each root tip was transferred to a clean slide, and 5 to 10 μL of 50% (v/v) acetic acid was applied. After degrading the root tip to fragments with a fine glass needle, a cover slip (18 × 18 mm) was applied. Squashing was performed by pressing firmly on the cover slip. The slide was immersed into liquid N_2, and the cover slip was flipped off with a razor blade and air dried.

Probe Labeling

For the in situ hybridization of the T-DNA, two different probes were used simultaneously. Plasmid pGEM (generously donated by J. Kooter, Vrije Universiteit, Amsterdam, The Netherlands) contained the *NPT II* sequence. Plasmid pJAc1 (generously donated by J.I. Yoder, University of Cologne, Germany) carried the *Ac* transposon. As a marker for chromosomes II and III, the 18S ribosomal DNA of soybean on plasmid pSR12B3 was used (generously donated by R.B. Meagher, University of Georgia, Athens, GA). For the in situ hybridization of the flavonol synthase gene, plasmid pCGP481 (generously donated by Florigene, Victoria, Australia) with the cDNA *Fl* clone was used. The probes were labeled by nick translation as reported by Montijn et al. (1994). For DNA gel blot analysis, a 4.3-kb *Ac* probe was prepared from plasmid SLJ4368 (provided by J.D.G. Jones, Sainsbury Laboratory, Norwich, UK), which was labeled using a commercial random-primed labeling kit (Amersham).

In Situ Hybridization

Slides were incubated with 100 μL of RNase A (100 μg/mL 2 × SSC for 60 min at 37°C under a cover slip in a humid chamber). Subsequently, the slides were rinsed three times for 5 min in 2 × SSC and 5 min in protease K buffer (20 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$) at 37°C and incubated in 100 mL of protease K solution (10 μg/100 mL) for 10 min at 37°C.

Slides were rinsed in PBS with 50 mM $MgCl_2$ for 5 min, fixed in 1% (v/v) formaldehyde in the same buffer for 10 min at room temperature, and rinsed in PBS for 5 min. The slides were dehydrated by submerging for 3 min in 70, 90, and 100% (v/v) ethanol and air dried. A 10-μL probe solution (5 ng/mL labeled DNA, 10% [v/v] dextran sulphate in HB50) was administered to the chromosome preparations, covered with a coverslip (18 × 18 mm), and denatured for 2 min at 80°C. Hybridization occurred overnight in a formamide humid chamber at 37°C. Posthybridization washes of the slides were done with SF50 (2 × SSC and 50% formamide, pH 7) three times for 5 min at 42°C. Slides were then rinsed briefly in 2 × SSC (42°C) and transferred to 0.1 × SSC at 56 to 58°C for stringent washing three times for 5 min.

Immunodetection of the slides was performed by rinsing in immunobuffer, which was 4 × SSC for biotin-labeled probes and TN (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) for digoxigenin-labeled probes. Slides were blocked with immunobuffer with 5 g/L nonfat dry milk (4 M) for biotin-labeled probes and with 0.5% (w/v) blocking reagent (TNB; Boehringer Mannheim) for digoxigenin-labeled probes. Biotin-labeled probe detection was performed with avidin-conjugated fluorescein isothiocyanate (FITC) (vector A2011) 1:1500 in 4 M and biotinylated anti-avidin D antibody (vector C0624) 1:100 in 4 M. Digoxigenin-labeled probe detection was performed in TNB with mouse anti-digoxigenin (Boehringer Mannheim 1333062) 1:500 in TNB, rabbit anti-mouse-conjugated FITC (Sigma F-7506) 1:1500 in TNB, and goat anti-rabbit-conjugated FITC (Sigma F-9262) 1:1500 in TNB. Slides with antibodies were incubated in a humid chamber at 37°C for 30 min. Between incubation with antibodies, the slides were rinsed three times for 5 min with immunobuffer with 0.05% (v/v) Tween 20. After immunodetection, slides were rinsed in PBS, dehydrated with 70, 90, and 100% (v/v) ethanol, and air dried. The chromosome preparations were then

mounted with 15-μL vectashield (Vector Laboratories, Inc., Burlingame, CA) with either 1 μg/mL propidium iodide or 500 ng/mL 4′,6-diamidino-2-phenylindole (DAPI), and a cover slip (32 × 24 mm) was applied. Control experiments were performed in which probe or antibodies were omitted to assure genuine detection of T-DNA sequences (results not shown).

Image Acquisition

Preparations were analyzed with a microscope (model BH2-RFC; (Olympus, Tokyo, Japan) equipped with a 100-W mercury lamp. Images were acquired with a cooled CCD camera (Astromed; AstroCam, Cambridge, UK). Metaphase spreads were scored for double spots on homologous chromosomes. Chromosome II was identified with simultaneous detection of labeled ribosomal DNA and by possession of a satellite. Direct fluorescence photographs were taken with Scotch 640-T ASA color slide film. Morphometric measurements were performed on an Apple Macintosh PPC7100 computer (Cupertino, CA) by using the program Object-Image (ftp://horus.sara.nl/norbert), which is a special version of National Institutes of Health Image (ftp://zippy.nimh.nih.gov). The length of the chromosome was measured using a polygon line, after which the position of the double spot was indicated. The position was then related to the whole chromosome length, giving a relative value between 0 and 1.

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