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Stable transfer of intact high molecular weight DNA into plant chromosomes

(*Agrobacterium*/plant transformation)

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ABSTRACT **In conjunction with an enhanced system for *Agrobacterium*-mediated plant transformation, a new binary bacterial artificial chromosome (BIBAC) vector has been developed that is capable of transferring at least 150 kb of foreign DNA into a plant nuclear genome. The transferred DNA appears to be intact in the majority of transformed tobacco plants analyzed and is faithfully inherited in the progeny. The ability to introduce high molecular weight DNA into plant chromosomes should accelerate gene identification and genetic engineering of plants and may lead to new approaches in studies of genome organization.**

The ability to stably transfer foreign DNA into plant chromosomes is the foundation of plant genetic engineering. DNA transfer to plants has been accomplished by many methods, including *Agrobacterium*-mediated transformation, biolistic transformation, and microinjection (1, 2). However, no method for routinely introducing DNA fragments larger than about 25 kb into the plant nuclear genome has yet been demonstrated. Genes with related functions, such as disease resistance genes in plants, have been found in clusters (3). A reliable system for transforming large segments (>100 kb) of DNA into plants would make it feasible to introduce a natural gene cluster or a series of previously unlinked foreign genes into a single locus. Such a group of genes could provide resistance to several different pests or pathogens, or it could constitute an entirely new metabolic pathway for production of a novel biomolecule. The integrated "megalocus" would be inherited as a single Mendelian unit and could easily be incorporated into conventional plant breeding programs. Large insert transformation would make it feasible to study the expression of plant genes or gene clusters in their native genomic context and might eliminate site-dependent gene expression, which can be a serious problem in plant transformation experiments. Finally, such a system may make positional cloning applicable to the isolation of genes that encode complex quantitative traits and allow for the transfer of one or more of these genes to various plant species (4).

The construction of large insert libraries in bacterial artificial chromosome (BAC) vectors has been reported for several plants (5–7) and animals (8, 9). These BAC libraries have average insert sizes of 100–180 kb, with inserts ranging from about 50 to 350 kb. BAC libraries are easier to construct, screen, and maintain than yeast artificial chromosome (YAC) libraries. While BAC vectors are designed for cloning large segments of DNA, they are not engineered for transformation of the cloned DNA back into plant genomes. *Agrobacterium tumefaciens* is a plant pathogen that transfers a portion of its genome into the plant chromosomes as part of its infection strategy. This natural gene transfer system has been engineered to deliver and integrate fragments of foreign DNA into plant chromosomes. In one report, when the transferred DNA (T-DNA) right border sequences of the *A. tumefaciens* Ti plasmid were inverted, 160 kb of the Ti plasmid DNA was unidirectionally transferred to a plant, *Kalanchoe tubiflorea* (10). A new form of BAC vector (termed binary-BAC or BIBAC) has been designed that is capable of replicating in both *Escherichia coli* and *A. tumefaciens* (C.M.H., unpublished results) and that has all of the features that are theoretically required for transferring large inserts of DNA into plant chromosomes, including the plant selectable markers neomycin phosphotransferase II (NPTII) for resistance to kanamycin and hygromycin phosphotransferase (HYG) for resistance to hygromycin. To assess the capability of the BIBAC to transfer large inserts to plants, BIBAC test constructs containing high molecular weight DNA inserts were introduced into several *A. tumefaciens* strains (C.M.H., unpublished results) and used to transform tobacco plants. The results of these experiments indicate that the BIBAC is capable of transferring at least 150 kb of foreign DNA, intact, into a plant nuclear genome. In addition, we show that the high molecular weight T-DNA is faithfully inherited in subsequent generations.

MATERIALS AND METHODS

Strains and Plasmids. UIA143 is a *recA*-deficient derivative of *A. tumefaciens* strain C58 (11). The plasmid pMOG101 is the disarmed (nontumorigenic) pTiB6 plasmid from *A. tumefaciens* strain MOG101 (12). The helper plasmids pCH30 and pCH32 (C.M.H., unpublished results) each carry *virG* from *A. tumefaciens* strain Bo542; pCH32 also carries *virE1* and *virE2* from *A. tumefaciens* strain A6. BIBAC test constructs contained a 30-kb yeast genomic DNA (BIBAC1.Y30) fragment or a 150-kb human genomic DNA (BIBAC2.H150) fragment and were introduced into several *A. tumefaciens* strains (C.M.H., unpublished work).

Plant Transformation. *Agrobacterium*-mediated transformation of tobacco was carried out as described by Horsch *et al.* (13). Potential transformed tobacco calli and regenerated plantlets were identified by selecting for resistance to kanamycin (300 mg/liter). Regenerated plants were assayed by polymerase chain reaction (PCR) (14). Primers used were as follows: NPTII foward, 5′-TCGGCTATGACTGGGCACAACAGA-3′; NPTII reverse, 5′-AAGAAGGCGATAGAAGGCGATGCG-3′; HYG foward, 5′-GATGTAGGAGGGCGTGGATATGTC-3′; and HYG reverse, 5′-CTTCTACA-

Abbreviations: BAC, bacterial artificial chromosome; BIBAC, binary-BAC; HYG, hygromycin phosphotransferase; NPTII, neomycin phosphotransferase II; T-DNA, transferred DNA.

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Table 1. Transformation of tobacco with BIBAC test construct BIBAC2.H150

Strain	*A. tumefaciens*	VirG/VirE	No. of resistant calli/no. of leaf strips inoculated	No. of transgenic plants	Efficiency, %
COR322	MOG101		0/60	0	—
			0/70	0	—
COR324	UIA143		0/75	0	—
	pMOG101		0/85	0	—
COR326	UIA143	VirG	11/80	9	11
	pMOG101		6/80	5	6
COR320	UIA143	VirG/VirE	17/72	16	22
	pMOG101		4/40	3	8

Data shown are from two independent experiments. Percent efficiency is defined as the number of transgenic plants (× 100) number of leaf strips used for cocultivation. The plants were counted as transgenic if they tested positive for NPTII by PCR; many of the plants were confirmed by Southern blot analysis. Care was taken to ensure that regenerated plants were unique (nonclonal) individuals.

CAGCCATCGGTCCAGA-3′. Thermal cycler conditions will be made available upon request.

Analysis of Tobacco Genomic DNA. Tobacco genomic DNA was prepared as described by Bernatzky and Tanksley (15). The DNA was quantified by comparing uncut genomic DNA to a standard curve. The ethidium-stained gel was captured to computer disc by using the EagleEye still video system and Eagle Sight application, both from Stratagene. The data were quantified by using IMAGECALC (16). Digested tobacco genomic DNA (approximately 8 μg for each sample) was separated on 0.9% agarose gels. Electrophoresis was carried out for 42–48 hr at 25 V in 1× NEB (100 mM Tris·HOAc/1 mM EDTA/12.5 mM NaOAc, pH = 8.1). DNA was transferred to Hybond N+ membranes by alkaline transfer using the protocol provided by the manufacturer (Amersham). The 150-kb, NPTII, and HYG probes will be described elsewhere. The NR probe is a PCR product generated using primers specific to the tobacco *nia-2* gene and pCLS16 (a tobacco *nia-2* cDNA clone) as template (17, 18). NR primer sequences and thermal cycler conditions will be made available upon request. All probes were labeled with [α-^{32}P]dCTP using the random hexamer primer method of Feinberg and Vogelstein (19). Southern blot hybridization and wash conditions were as described by Bernatzky and Tanksley (15). Blots were visualized by using a PhosphorImager and IMAGEQUANT, both from Molecular Dynamics, and manipulated by using Adobe Photoshop (Adobe Systems, Mountain View, CA).

RESULTS AND DISCUSSION

Transformation of Tobacco with BIBAC Test Constructs. Table 1 shows the data from two independent experiments in which four different *A. tumefaciens* strains containing BIBAC2.H150 were used to transform tobacco. Of these *A. tumefaciens* strains, only those enhanced with additional copies of VirG or VirG/VirE produced transgenic tobacco plants. The VirG protein activates expression of the virulence genes that effect the transfer of the T-DNA from *Agrobacterium* to the plant cell nucleus (20). VirE2 is a single-stranded DNA-binding protein that may protect the T-DNA from degradation by endonucleases and may be involved in nuclear uptake of the T-DNA (21). For a review on *A. tumefaciens* virulence genes and the process of T-DNA transfer see Zupan and Zambryski (22). That no kanamycin-resistant calli were obtained for *A. tumefaciens* strains COR322 and COR324 may, in part, be due to the fact that *A. tumefaciens* contamination was consistently a problem with these strains. Higher concentrations (OD_{600} = 0.5–1.0) of *Agrobacterium* cells improved the efficiency of transfer for the strains containing the additional helper plasmids pCH30 or pCH32. Cultures at lower concentrations reduced or eliminated the contamination for strains COR322 and COR324 but did not produce any transformed plants. It is expected that the additional copies of the VirG protein result in the increased expression of all the *vir* genes. This shift in the metabolism of *A. tumefaciens* may also reduce the growth rate of the bacteria during the transformation process.

Putative transgenic plants that regenerated shoots and roots on selective media were subsequently analyzed by PCR using

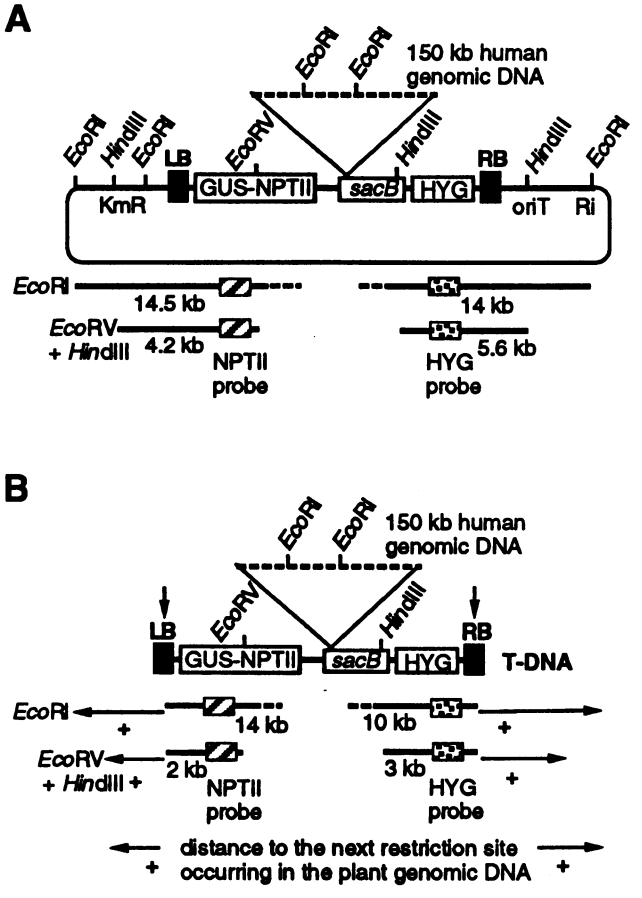

FIG. 1. Schematic description of the strategy used to detect and characterize T-DNA inserts in tobacco plants transformed with BIBAC2.H150. (*A*) Restriction fragments that hybridize to NPTII and HYG probes for BIBAC2.H150. (*B*) Restriction fragments that hybridize to NPTII and HYG probes in the genomic DNA isolated from transgenic plants. During the process of T-DNA transfer, the T-DNA is cleaved in the border sequences, transferred to the plant cell, and subsequently integrated into the plant nuclear genome. Because of the cleavage and integration events, the sizes of the plant genomic DNA fragments that hybridize to the NPTII and the HYG probes will be different from the sizes of the BIBAC2.H150 fragments that hybridize to the same probes. GUS, β-glucuronidase.

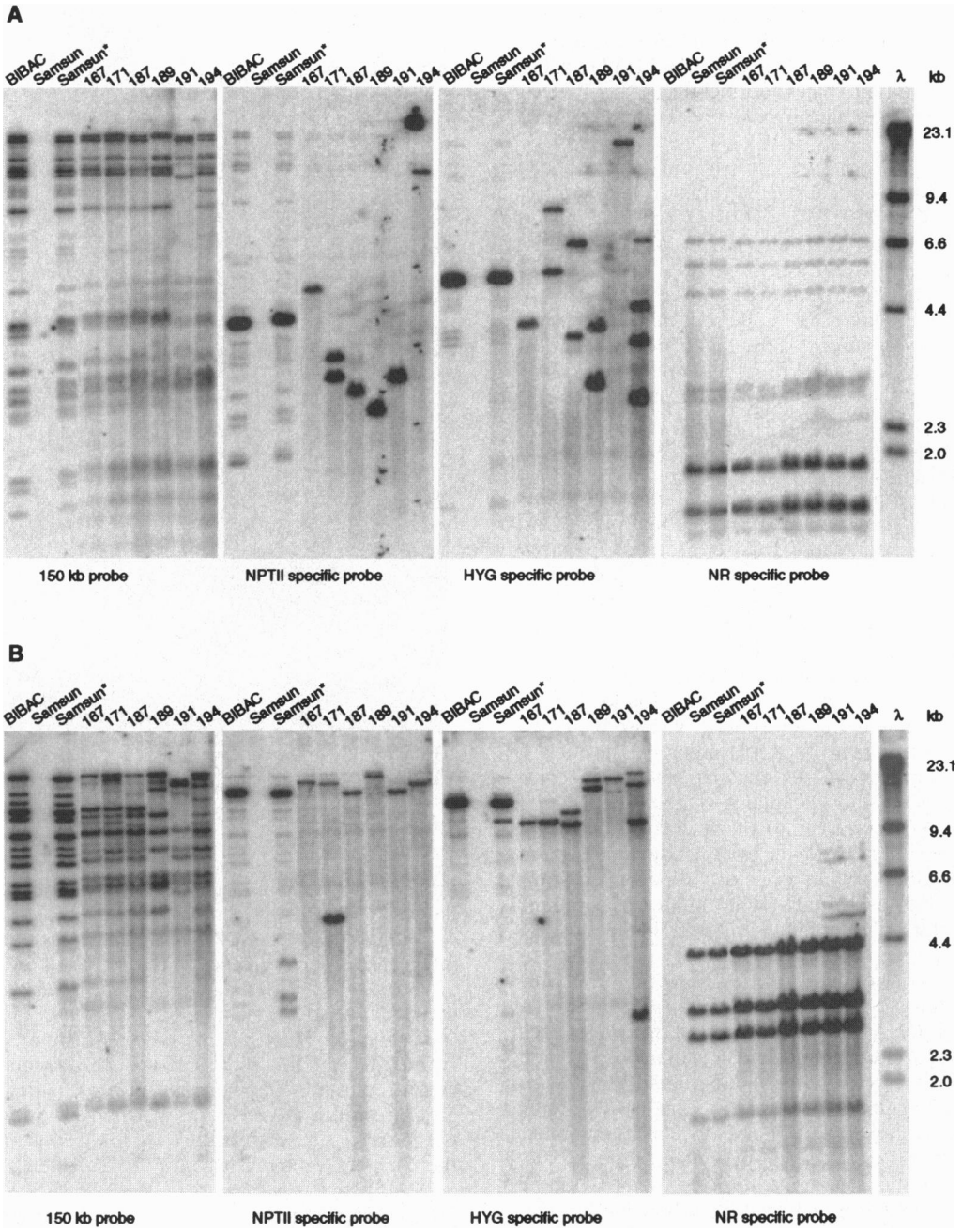

FIG. 2. Analysis of tobacco genomic DNA from R_0 tobacco plants transformed with BIBAC2.H150. (*A*) All samples were digested with *Eco*RV and *Hin*dIII. The NPTII and HYG probes hybridize to restriction fragments that include the flanking plant genomic sequences but do not extend into the 150-kb insert. (*B*) All samples were digested with *Eco*RI. The restriction fragments that hybridize to the flanking probes also extend into the 150-kb insert. That is, when the 150-kb insert is used as the probe, we expect two plasmid BIBAC2.H150 bands to be absent from the fingerprint of the R_0 plants and two different bands to appear. The two bands that change are predicted to correspond to the plant genomic sequences flanking the insertion and should hybridize to the left (NPTII) and right (HYG) border probes. BIBAC refers to plasmid BIBAC2.H150. Samsun refers to tobacco var. Samsun NN and is a wild-type (nontransgenic) plant. Samsun* is the same wild-type tobacco genomic DNA "spiked" with plasmid BIBAC2.H150. BIBAC2.H150 DNA (1.2 ng) was loaded at 4× the tobacco genomic equivalent. Samsun NN DNA was "spiked" with the same amount of BIBAC DNA as in the BIBAC sample (1.2 ng). To compensate for this discrepancy, a lower exposure of the first three lanes of the images is presented. Each number—167, 171, 187, 189, 191, and 194—refers to a unique R_0 tobacco plant. λ indicates λ phage DNA digested with *Hin*dIII as size markers.

primers specific to the plant selectable marker genes NPTII and HYG. Whereas 71% (10/14) of the plants transformed with COR326 and 58% (11/19) of the plants transformed with COR320 tested positive by PCR for both NPTII and HYG, the remainder were positive for NPTII but not for HYG. Because NPTII and HYG flank the 150-kb insert, a positive assay for both suggests that the entire region had been transferred.

Analysis of Transgenic Tobacco Plants. To determine whether the entire 150-kb human genomic insert was transferred intact into the plant chromosomes, tobacco genomic DNA was isolated from the transgenic tobacco plants, cut with restriction endonucleases, and analyzed by hybridization with various probes. Tobacco genomic blots were probed consecutively with (*i*) the 150-kb human insert, (*ii*) a NPTII-specific probe (left border), (*iii*) a HYG-specific probe (right border), and (*iv*) a probe for the single-copy nitrate reductase (NR) gene to show the quality and quantity of each tobacco genomic DNA sample on the blot. During the transfer process, the

T-DNA is cleaved within the left and right border sequences. When hybridized to plant genomic DNA that has been digested with restriction endonuclease(s), the NPTII and HYG probes identify the fragments that span the junction of the T-DNA and the flanking plant genomic DNA. Fig. 1 illustrates this analysis.

The first group of regenerated transgenic (R_0) tobacco plants that were analyzed had been transformed with *A. tumefaciens* strain COR320 (see Table 1) and had tested positive by PCR for both NPTII and HYG. When the plant genomic DNA was digested with restriction enzymes *Eco*RV and *Hin*dIII as shown in Fig. 2*A*, or *Eco*RI as shown in Fig. 2*B*, hybridization to the 150-kb insert probe revealed that four of the plants had a distinct pattern of fragments nearly identical to that of BIBAC2.H150. We interpret the more intense bands of the fingerprint to be due to the comigration of several fragments, each of which hybridizes to the probe, thereby increasing the intensity of the signal. Note that there is no detectable cross-hybridization of the human genomic insert to the genomic DNA prepared from the wild-type (untransformed) Samsun NN tobacco plant. Two other plants had some fragments in common with BIBAC2.H150 but appeared to have undergone deletion of a portion of the insert. Two additional plants (numbers 189 and 191) were analyzed but are not shown in Fig. 2. One appeared to have an intact insert, whereas the other was partially deleted. In total, of eight independent R_0 transformed tobacco plants analyzed, five (63%) appeared to contain the entire intact 150-kb human DNA fragment. Although this evaluation is based on two different restriction enzyme profiles, it is impossible to rule out that small rearrangements or deletions may have occurred in the T-DNA. Transgenic tobacco plants that had 150 kb of human genomic DNA inserted into the nuclear genome are shown in Fig. 3. These plants were phenotypically wild type and most were fertile. Similar plant transformation experiments using BIBAC1.Y30 demonstrated that the 30-kb yeast genomic insert is also transferred completely and remains intact (unpublished data).

The various sizes of the restriction fragments identified by hybridization to the NPTII- or the HYG-specific probes indicated that the T-DNA integrated into a unique site in each transformed tobacco plant. Several plants that appeared to contain a single intact copy of 150-kb human DNA also displayed multiple fragments hybridizing to the HYG-specific right border probe. However, only one of those plants (number 171) also showed two fragments that hybridize to the NPTII-specific left border probe. These results are consistent with the concept that the T-DNA is transferred linearly from the right border to the left border, and therefore resistance to kanamycin should be indicative of complete transfer. Additional copies of the right border sequences, as indicated by the HYG probe, would be present if transfer of the T-DNA was initiated but not completed. Certainly, it is not surprising that all of the integrated T-DNA transfers are not complete. Schröder *et al.* (23) reported the analysis of 43 plants transformed with a binary vector containing a much smaller (5.3-kb) T-DNA. These transgenic plants showed variation in the number of integrated right and left T-DNA border fragments as well as tandem and inverted repeats of the T-DNA. In fact, only 22% of the progeny segregated both marker genes as a single locus. Initial results suggest that the integration events generated by the BIBAC transfer of large segments of DNA may not be as complex as those produced by standard binary vectors. This may be because replication and transfer of the T-DNA are coupled and because the BIBAC is a single-copy plasmid in *Agrobacterium*, whereas most commonly used binary vectors are present in 5–10 copies per cell. The BIBAC system is currently being evaluated in tomato, and preliminary results indicate that the 150-kb test construct is also transferred intact into tomato chromosomes in R_0 transformants (unpublished data).

FIG. 3. Transgenic tobacco plants, including several transformed with BIBAC.H150.

Analysis of Tobacco Progeny. To assess the stability of the integrated T-DNA in the transgenic plants, R_1 progeny from 13 individual self-pollinated R_0 plants containing the 150-kb insert were assayed for segregation of the 150-kb human transgene by first screening for resistance to kanamycin. Approximately 50–100 R_1 seeds were germinated on selective media containing kanamycin, and the numbers of healthy green resistant (Kan^R) and bleached yellow sensitive (Kan^S) progeny were recorded. Because tobacco is an allotetraploid, the R_0 hemizygous transgenes are expected to segregate in a 3 (Kan^R) to 1 (Kan^S) ratio. For 11 of the R_0 plants, the R_1 progeny segregated as expected. The R_1 progeny of the other two R_0 plants segregated at a ratio significantly different ($P \leq 0.05$) from a 3:1 ratio but were consistent with the expected ratio (15:1) for two unlinked insertion events (unpublished data). Segregation ratios were statistically tested, using the χ^2 goodness of fit to the expected ratio.

Fig. 4 shows that the transferred DNA is inherited intact in each of four R_1 plants derived from a single R_0 parent. Six (Kan^R) R_1 plants from four different R_0 parents were analyzed by PCR for the NPTII and HYG genes. In every case (24 plants total) the PCR results were identical to results for the R_0 parent. Notably, in the cases where the progeny of R_0 plants with incomplete 150-kb inserts were analyzed (unpublished data), the deleted version of the insert was also faithfully transmitted to the R_1 plants without further changes. This result suggests that if any deletions or rearrangements occur in the T-DNA, they probably occur during the transfer process. Once integrated, the transferred DNA becomes fixed and is stably transmitted to subsequent generations.

A binary-BAC (BIBAC) vector is shown to be capable of transferring at least 150 kb of foreign eukaryotic DNA into plant chromosomes. These results open up a number of new

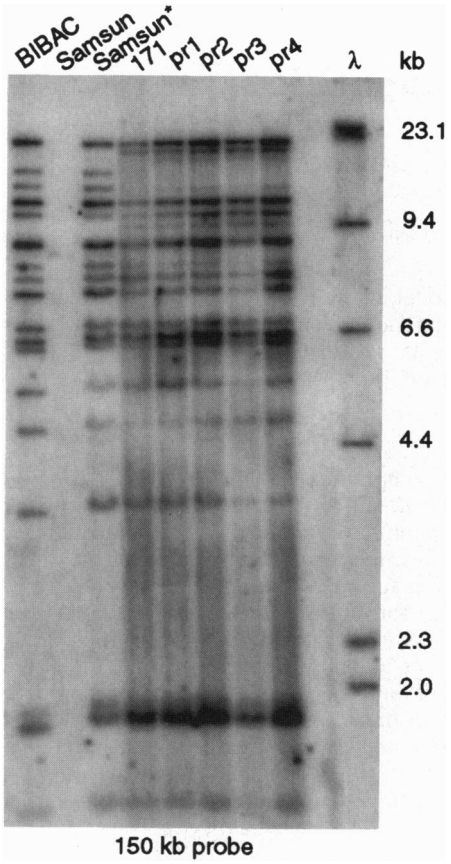

FIG. 4. Southern blot analysis of tobacco genomic DNA from R_1 tobacco plant 171. All samples were digested with *Eco*RI. BIBAC refers to BIBAC2.H150. Samsun refers to tobacco var. Samsun NN and is a wild-type (nontransgenic) plant. Samsun* is the same wild-type tobacco genomic DNA "spiked" with plasmid BIBAC2.H150. BIBAC2.150H DNA (0.3 ng) was loaded at 1× the tobacco genomic equivalent. Samsun NN DNA was "spiked" with the same amount of BIBAC DNA as in the BIBAC sample (0.3 ng). Individual progeny are designated pr1-4.

possibilities for plant molecular biology and for genetic engineering of crop plants. This system should streamline the positional cloning and transfer of desirable traits into agronomically important plants and reduce the concomitant introduction of deleterious traits. It will make it feasible to transform entire genomic libraries into plants to identify desirable characteristics by complementation even without map-based cloning and will facilitate the identification of plant quantitative trait loci. In addition, the BIBAC system may be used to characterize regulatory sequences and/or genes from other eukaryotic systems in an *in vivo* plant system.

The introduction of large domains of DNA into plant chromosomes may lead to integration site-independent gene expression, as has been reported in mammals (24, 25). With the advent of the BIBAC system, it may now be possible to clone large enough segments of DNA to study the composition and function of huge structural elements such as heterochromatin and plant centromeres. New approaches to studies of homologous recombination and gene silencing may also be feasible. In addition, it will be possible to introduce large regions of DNA from heterologous systems to expand studies of eukaryotic genome organization and maintenance.

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