Published January, 1989

Inheritance and Expression of Genes for Kanamycin and Chloramphenicol Resistance in Transgenic Cotton Plants

Paul Umbeck,* Will Swain, and Ning-Sun Yang

ABSTRACT

To fully evaluate and characterize the stability of traits transferred via *Agrobacterium* transformation, foreign gene expression must be examined in sexually derived progeny. The objective of this study was to analyze three transgenic cotton (*Gossypium hirsutum* L.) plants (no. 1397, no. 1427, and no. 1438) for inheritance and expression of their foreign genes. Following regeneration from tissue cultures transformed using *A. tumefaciens*, primary regenerated plants were tested for expression of T-DNA marker genes encoding the proteins neomycin phosphotransferase II (NPT-II) and chloramphenicol acetyltransferase (CAT). To test for transmission of marker traits flowers were either self-pollinated or crossed to the cotton cultivar Coker 312. Selfed progeny and backcrosses were germinated in vitro and selected on medium containing 50 mg L^{-1} kanamycin. At the three-leaf stage of development, enzymatic activity of NPT-II and CAT was measured on seedlings capable of germination and survival in the presence of kanamycin. Progeny derived from selfed and backcrossed pollinations of plants no. 1397 and no. 1427 both segregated 3:1 and 1:1, respectively, as expected for a single gene trait. No CAT activity was detected in these plants or their progeny. Southern blot analysis of regenerated plants, selfed progeny and backcrosses of no. 1397 and no. 1427 confirmed the inheritance of only the NPT-II coding sequence portion of the T-DNA. In plant no. 1438 and its backcross progeny, enzyme expression of both NPT-II and CAT was detected. Segregation analysis of no. 1438 backcross progeny showed a 1:1 pattern of inheritance. Southern blot analysis of no. 1438 and its progeny showed that the entire T-DNA coding region was present. Despite the presence and function of the CAT gene in backcross progeny of no. 1438 these plants did not display chloramphenicol resistance. Germination of seeds obtained from no. 1397, no. 1427, and no. 1438 on kanamycin-containing (50 mg L^{-1}) medium efficiently selected for transgenic seedlings expressing kanamycin resistance. Our data support the conclusion that the single gene insertion of foreign traits in cotton is inherited and expressed in a Mendelian fashion.

THE TRANSFORMATION of higher plants has been accomplished by several different methods (11). Plants in which the inheritance of foreign genes has been demonstrated were derived either by *Agrobacterium* infection, protoplast cocultivation or direct DNA transfer into protoplasts. In dicotyledonous species the most efficient method has been the use of nononcogenic *Agrobacterium* strains (11). This technique has been especially important in species such as tobacco (*Nicotiana tabacum* L.) where plant regeneration is obtained by organogenesis (14).

Transformation can be confirmed by analysis of the primary regenerants for foreign DNA integration and its expression tested by the presence of protein or RNA encoded by introduced foreign genes. Expression of other traits can be evaluated directly by bioassays. For example, regenerated plants could be sprayed for detection of herbicide resistance (6), inoculated with pathogenic organisms to test for disease development (1), or subjected to insect feeding to test for expression of a toxin (2).

Several recent studies have reported the inheritance of foreign genes in plants obtained by using nonvirulent strains of *Agrobacterium* (5,7,10) or protoplast transformation (18). Most studies have used tobacco as the host plant and all have demonstrated that dominant selectable markers such as kanamycin resistance are inherited in a Mendelian fashion. In most circumstances, foreign genes can be transmitted to progeny with a high degree of fidelity (5, 17). The degree of stability can potentially be influenced by the site of integration, or by structural changes in the DNA during the transformation process. These structural changes can occur as rearrangements, amplification, deletions, or concatermerization (19,22). Since foreign genes are randomly inserted into the plant genome, the site of integration could influence the relative stability of genes if certain regions of the chromosome are more likely to undergo recombination. While it is not clearly understood why different sites of foreign DNA integration influence the amount of transcription and translation, the result is commonly referred to as a position effect (7,15). Different lines of trans-

Agracetus, 8520 University Green, Middleton, WI 53562. Contribution of Agracetus. Received 24 Feb. 1988. *Corresponding author.

Published in Crop Sci. 29:196–201 (1989).

genic plants might show varying degrees of stability and expression of their newly acquired traits (2,5,8).

Using a nononcogenic *Agrobacterium* vector, we transformed cotton cells, selected for antibiotic resistance, and regenerated fertile plants (27). The modified T-DNA consisted of two marker genes encoding neomycin phosphotransferase II (NPT-II) and chloramphenicol acetyltransferase (CAT) that specify resistance to the antibiotics kanamycin and chloramphenicol, respectively. Both genes were regulated by the nopaline synthase promoter, which is constitutively expressed in plant cells. In this report we provide evidence for stable monogenic inheritance of the bacterial marker genes in transformed cotton.

MATERIALS AND METHODS

Plant Materials

Seeds of Coker 312 were kindly provided by H. Webb (Coker's Pedigreed Seed Co., Hartsville, SC). Tissue culture explants were obtained from the hypocotyl region of 4-d-old germinated seedlings. Tissue culture and plant regeneration techniques were performed as described by Trolinder and Goodin (26). Vector construction, transformation, and recovery of the transgenic plants no. 1397, no. 1427, and no. 1438 were previously described by Umbeck et al. (27). Southern blot data of the primary regenerants previously confirmed that two of the plants, no. 1397 and no. 1427, carried a portion of the modified T-DNA that contained only the NPT-II gene. The third plant, no. 1438, contained the full-length T-DNA insertion and expressed both the NPT-II and CAT genes.

Regenerated plants were maintained under greenhouse conditions and grown to maturity. Plants were fertilized weekly [using Peters 20–10–20 Peat-Lite-Special (W.R. Grace and Co., Memphis, TN)] and watered with reverse osmosis water. Temperature and photoperiod were 26 °C day and 21 °C night on a 16-h photoperiod. Incidental sunlight was supplemented with high-pressure sodium lamps (Energy Techniques, York, PA) with General Electric bulbs. Seed increases of transgenic plants no. 1397 and no. 1427 were supplemented by making cuttings of the regenerated plant. New shoots, 7.5 to 10 cm long, were dipped in Hormex 1 (Brooker Chemical, Hollywood, CA) rooting powder and rooted in a 1:1 mixture of peat and turface (calcined clay). Cuttings were maintained under mist for about 2 wk before repotting. Plants and cuttings of no. 1397 and no. 1427 were self-pollinated and backcrossed as male parents onto plants of Coker 312. Plant no. 1438 was determined to be male-sterile based on its inability to set seed after self-pollination; however, progeny were obtained when no. 1438 was used as a female parent and backcrossed with Coker 312. In this report, regenerated plants, selfed progeny and backcross progeny refer to the primary regenerated plant derived from somatic embryos, the first generation obtained after selfing a regenerated plant, and the first generation recovered after backcrossing a regenerated plant, respectively. A chi-square analysis was performed on selfed and backcrossed progeny segregating for kanamycin resistance to test various genetic models of inheritance.

In Vitro Seedling Assay

Harvested seeds were prepared for germination according to a scaled-down procedure of McCarty (16). Seeds were acid delinted in concentrated sulfuric acid, rinsed in cold water, then neutralized in a saturated solution of sodium bicarbonate and rinsed again in water. Delinted seeds were allowed to dry at room temperature. Prepared seeds were scarified at the chalazal end wounding the cotyledons, then sterilized using 30 g L^{-1} sodium hypochlorite and rinsed three times with sterile distilled water. Sterilized seeds were soaked overnight in a solution containing 50 mg L^{-1} kanamycin and plated onto Stewart and Hsu medium (25) containing the same concentration of kanamycin. After 10 d of incubation in a tissue culture room at 28 °C and a 16-h photoperiod (200 μmol m^{-2} s^{-1}), seedlings were evaluated for resistance to the antibiotic. Seedlings were photographed 7 to 10 d after germination.

Expression Assay for NPT-II and CAT Activity

Plant tissue (100 mg sample^{-1}) from seedlings at the three-leaf stage of development of Coker 312, regenerated plants, and selfed progeny and backcrosses was extracted in 150 μL of buffer solution (100 mg L^{-1} bromophenol blue, 200 g L^{-1} glycerol, 128 m*M* beta-mercaptoethanol, 125 m*M* tris-HCl at pH 6.8, 2 g L^{-1} sodium dodecylsulfate, and 30 μg bovine serum albumin sample^{-1}) as reported by Sanders et al. (23). Neomycin phosphotransferase II analysis was carried out as described by Reiss et al. (21). Purified NPT-II enzyme, supplied by D. Gelfand, Cetus Corporation (Emeryville, CA), was used as a standard. Purified CAT enzyme was purchased from Pharmacia (Piscataway, NJ). Protein quantitation was carried out according to the procedure of Bradford (3). Extracts for CAT assays were prepared as described by Herrera-Estrella et al. (13) and CAT activity determined by the method of Gorman et al. (12).

Southern Blots

The DNA was prepared from frozen leaf tissue by the method of Dellaporta et al. (9). It was digested with restriction endonucleases under conditions recommended by the supplier (New England Biolabs, Beverly, MA), resolved by electrophoresis in an 0.8% agarose gel, and transferred to nylon membranes (ICN, Irving, CA) as described by Southern (24). Phosphorus-32 labeled RNA hybridization probes were synthesized in vitro using an SP6 transcription system (Promega Biotec, Madison, WI) and (α-^{32}P) cytidine triphosphate (CTP) (approximately 15 TBq $mmol^{-1}$, Amersham, Arlington Heights, IL). The template used, produced a run-off transcript corresponding to the minus strand of the NPT-II coding region. Reaction conditions were those recommended by the supplier and employed 3.7×10^{12} Bq of radiolabeled CTP. Incorporation of the labeled nucleotide was typically 70 to 80%. Hybridization and washing conditions were as described by Church and Gilbert (4). Hybridizing fragments were detected by autoradiography at −80 °C using X-Omat AR5 film (Kodak, Rochester, NY) with two intensifying screens (Cronex Lightning Plus, DuPont, Wilmington, DE).

RESULTS AND DISCUSSION

We have previously described the regeneration and characterization of transgenic cotton plants (27). To examine inheritance of the kanamycin and chloramphenicol genes, a second generation of progeny plants was obtained. Regenerated plants were self-pollinated and backcrossed to Coker 312. Transmission of the kanamycin resistance trait and expression of chloramphenicol acetyltransferase was initially examined by assaying leaf extracts of all progeny seedlings. Based on the presence or absence of enzyme activity, it was determined which plants inherited a functional gene copy of NPT-II or CAT.

An NPT-II assay of a sample of progeny obtained from selfing plant no. 1427 is shown in Fig. 1. Positive transformants were clearly distinguishable in the population. Ability to discriminate between resistant and sensitive seedlings allowed us to follow the segregation of the newly acquired kanamycin resistance traits. Segregation classes were tabulated and a chi-square analysis for the expected ratio was calculated (Table 1). The expected ratio for single gene inheritance in selfed and backcrossed progeny was observed for progeny from all three regenerated plants.

While we were able to obtain only a few seed from self-pollinations of plant no. 1438, reciprocal backcrosses with the source cultivar of Coker 312 as the male parent were quite successful. The backcrossed progeny were fertile and set seed normally upon self-pollination. Thus, it appeared that the partial self-sterility associated with this plant was transient and probably due to carry-over effects of the tissue culture process. Expression of marker genes in backcross progeny derived from the cross no. 1438 × Coker 312 was evaluated by NPT-II and CAT assays. Results of the NPT-II and CAT assays are included in Table 1. For this type of mating, the presence or absence of NPT-II activity was inherited in a 1:1 segregation ratio. Expression of the unselected CAT gene also segregated 1:1. An illustration of this analysis is shown in Fig. 2. The progeny clearly segregated for positive (Fig. 2, lanes 5,6,7, and 9) or negative CAT activity (lanes 3,4,8, and 10). All offspring that gave CAT activity were also positive for NPT-II activity. Thus, the full-length T-DNA with the linked marker enzymes was inherited intact.

Using the current method of NPT-II electrophoretic analysis, we (20) have found that crude plant cell extracts contain protein kinase-like, nonspecific activities. Similar protein phosphorylating nonspecific activities have been observed by other investigators (21,29). These bands can be observed in the upper portion of the gel in Fig. 1 and 3. Trypsin treatment of a gel containing nonspecific activity will result in the disappearance of these bands. None of the upper bands appeared dependent on the presence of NPT-II because they also appeared in gels, which were not treated with neomycin sulfate in the indicator gel (20). Similar activities also have been observed in samples of nontransformed regenerated plants.

The remaining progeny were screened by using an in vitro seedling growth assay. Kanamycin-imbibed seeds were plated onto Stewart and Hsu medium containing 50 mg L^{-1} kanamycin. Both kanamycin insensitive and sensitive seeds were able to germinate, but only the resistant seedlings continued to grow. After 5 to 7 d of growth, susceptible seedlings showed chlorotic lesions on the cotyledons and lacked lateral root development. Cotyledons of kanamycin-sensitive cotton never became completely chlorotic, but instead showed irregular yellow areas. This is in contrast to the complete chlorosis observed with tobacco seedlings when germinated in the presence of kanamycin (18). Two weeks after germination most of the sensitive plants had died due to development of a necrotic zone in the hypocotyl region. All kanamycin-susceptible plants were dead 3 to 4 wk after germination.

Table 1. Segregation of neomycin phosphotransferase II and chloramphenicol acetyltransferase expression in selfed and backcrossed progeny derived from transgenic cotton plants.

	Number of plants						
	NPT-II		CAT				
Cross†	(+)	(−)	(+)	(−)	Ratio tested	X^2 Value	P
no. 1397 ⊗ Coker 312 ×	20	7	0	0	3:1	0.01	<0.95
no. 1397	67	68	0	0	1:1	0.01	<0.90
no. 1427 ⊗ Coker 312 ×	104	35	0	0	3:1	0.01	<0.95
no. 1427	71	66	0	0	1:1	0.18	<0.50
no. 1438 × Coker 312	13	10	NT‡	NT	1:1	0.39	<0.75
no. 1438 × Coker 312	NT	NT	14	17	1:1	0.29	<0.75

† × Symbol indicates progeny tested were obtained from selfed pollinations. For all backcross pollinations the female parent is listed first and the parental cultivar was Coker 312.
‡ NT Indicates not tested.

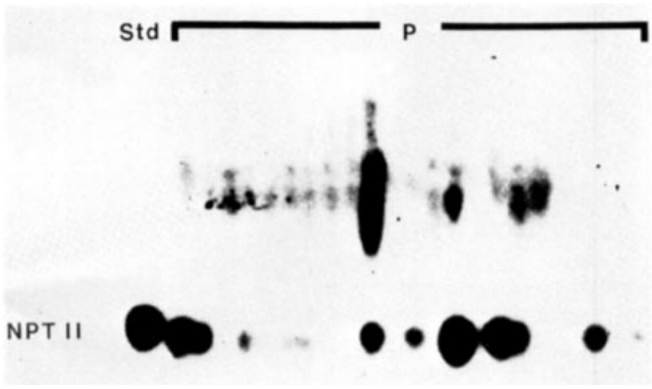

Fig. 1. Neomycin phosphotransferase II assay of cotton leaf extracts from selfed progeny obtained from plant no. 1427. Standard (Std) is purified NPT-II enzyme, which has a molecular weight of 27 500 daltons. Each of the remaining 22 lanes (P) is a sample from different selfed progeny plants.

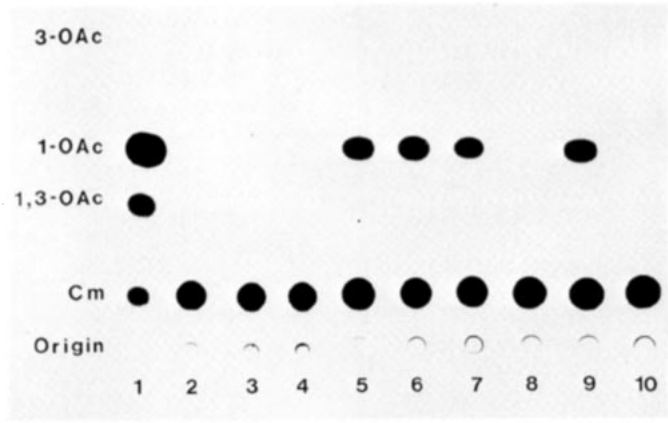

Fig. 2. Chloramphenicol acetyltransferase assay of cotton leaf extracts of nonselected backcross progeny obtained from no. 1438 × Coker 312. The positions of the origin of the chromatogram, chloramphenicol (Cm), 1-acetylchloramphenicol (1-OAc), 3 acetylchloramphenicol (3-OAc) and 1,3-diacetylchloramphenicol (1,3-OAc) are indicated. Lane 1 has 0.9 units of purified enzyme added to a nontransformed plant leaf extract; lane 2 is a leaf extract derived from a nontransformed regenerated plant; lanes 3 to 10 are extracted leaf samples of progeny.

Resistant seedlings did not develop any chlorotic lesions and continued to undergo normal plant development although their rate of root growth was slower than the untreated control (Fig. 4).

Confirmation of NPT-II expression in progeny was performed by enzymatic assay of leaf extracts (Fig. 3). All plants were of similar age and development. The tissue source was the first or second true leaf. All kanamycin-resistant progeny were positive for the NPT-II enzyme. Thus, the kanamycin-containing medium efficiently selected for NPT-II expressing plants.

The organization of the NPT-II sequences present in the transformed cotton plants was analyzed by Southern blots. An analysis of plant no. 1427 and several of its self-pollinated progeny is shown in panel C of Fig. 5. Panels A and B indicate the restriction maps of the modified T-region found in pCMC1204 and plant no. 1427, respectively. Panel B was constructed from numerous Southern blots (data not shown) employing the enzymes indicated on the map. As noted previously, plant no. 1427 contains only a portion of the modified T-DNA from pCMC1204. Consequently, the *Hind* III digest shown in panel C of Fig. 5 produces a fragment from plant no. 1427 (lane 5), which is larger than the internal *Hind* III fragment from the T-region of pCMC1204 (lane 2). Thus, the fragment from plant no. 1427 extends from the *Hind* III site immediately upstream of the NPT-II coding region, across the junction formed by integration into the cotton genome, to another *Hind* III site in cotton DNA. The location of the junction with cotton DNA has not been mapped exactly, but is less than 100 base pairs distal to the NPT-II *Sal* I site. The structure suggests that the attenuated T-region in plant no. 1427 may have resulted from a homologous recombination between the nos-polyadenylation regions of the NPT-II and CAT genes. Plant no. 1438 contains the entire modified T-DNA (data not shown) demonstrating that such structures can be propagated in *Agrobacterium*, transferred to cotton, and integrated and stably maintained in the cotton genome. The T-regions that include large repeated sequences (in this case direct repeats) may be less stable than those that do not, particularly in the bacteria.

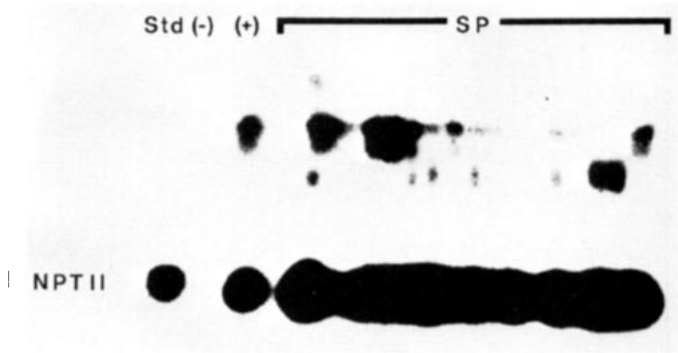

Fig. 3. Neomycin phosphotransferase II (NPT-II) assay of cotton leaf extracts of selected selfed progeny from plant no. 1427. Standard is purified NPT-II enzyme. Positive (+) and negative (−) control lanes are extracts derived from transformed plant no. 1427 and a nontransformed regenerated plant, respectively. The remaining 18 lanes (SP) are samples from selfed progeny selected on 50 mg L^{-1} kanamycin.

Detection of only one junction fragment in plant no. 1427 indicates that this plant contains a single insertion of the NOS-NPT-II gene. Lanes 5 to 16 in panel C of Fig. 5 show that this gene is stably transmitted to the progeny. A second, larger fragment apparent in lane 12 of this blot is due to incomplete digestion because both digestion of the same DNA with other restriction endonucleases and other digests with *Hind* III give patterns identical to that observed for plant no. 1427. Out of 22 progeny analyzed, 16 contained the expected hybridizing fragment and showed NPT-II enzyme activity.

Our results have demonstrated the Mendelian inheritance of foreign genes in cotton. We have shown that transformed cotton seedlings can be readily selected on kanamycin-containing medium. Both enzyme assays and DNA analysis confirmed that seedlings resistant to kanamycin were transformed while nontransformed seedlings were sensitive to the antibiotic. Studies to further evaluate the meiotic stability of these and other foreign genes are in progress. It is expected that some transgenic plants will not offer such straightforward interpretation of the effects of transformation on cotton. For example, where extended periods of tissue culture are required, somaclonal variation may have an increasingly important impact on foreign gene expression and organization. The study reported here, however, indicates that transformation of cotton should provide opportunities to improve upon disease and insect resistance, seed quality, and fiber properties.

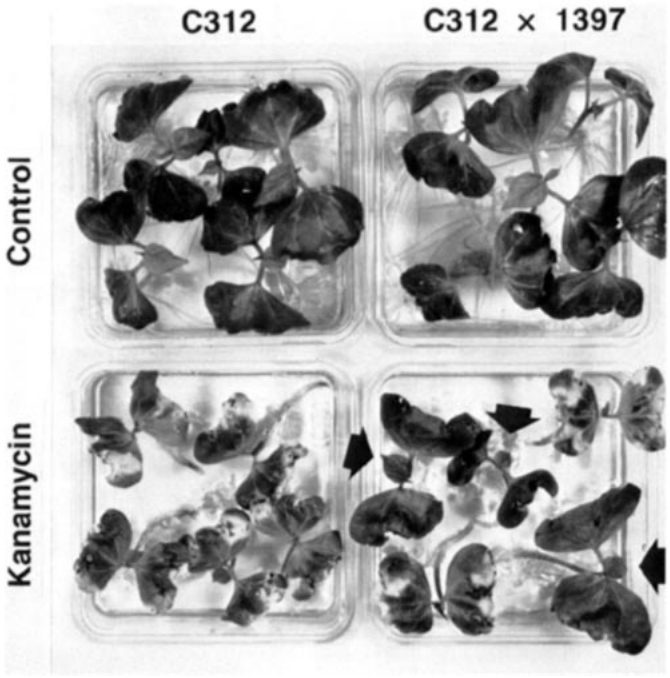

Fig. 4. Segregation of kanamycin resistance in cotton backcross progeny from Coker 312 × 1397. Coker 312 is the explant source cultivar and is abbreviated as C312. Plant no. 1397 is a regenerated transgenic plant previously shown to contain the neomycin phosphotransferase II gene. Control medium contains Stewart and Hsu salts (24) and vitamins. Kanamycin medium contains the same components plus 50 mg L^{-1} kanamycin. Arrows indicate resistant seedlings.

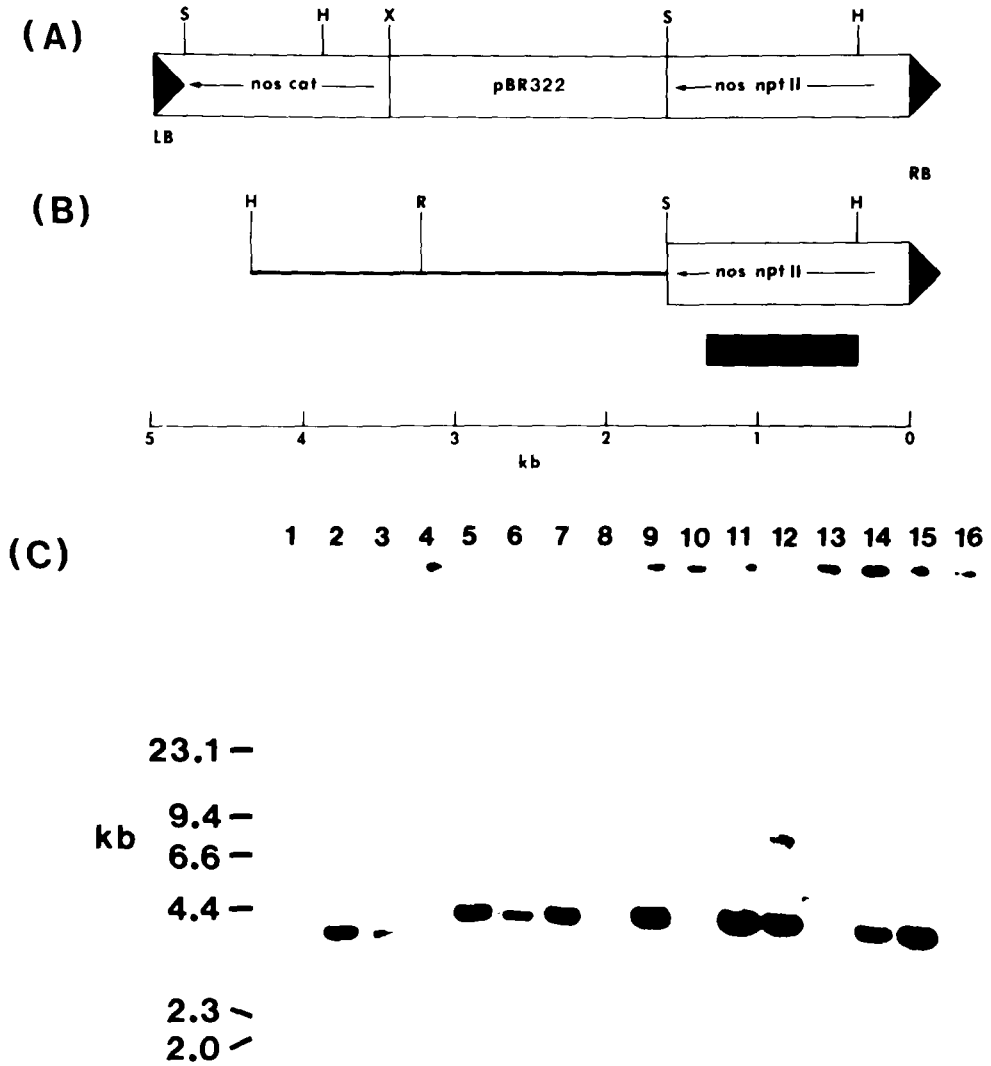

Fig. 5. Southern blot hybridization to neomycin phosphotransferase II sequences in transformed cotton plants. Restriction maps of the modified T-region of pCMC1204 (panel A) and the portion of this region found in plant no. 1427 (panel B) are shown. The modified T-DNA including the neomycin phosphotransferase II (NOS-NPT-II) and chloramphenicol acetyltransferase (NOS-CAT) genes is indicated. RB and LB indicate the right and left border repeats, respectively. The solid line in (B) indicates cotton DNA flanking the T-DNA insertion. The direction of transcription of the NOS-NPT-II and NOS-CAT genes is shown by an arrow. The bar in (B) indicates the hybridization probe used in (C). DNA was digested with *Hind* III. Samples in each lane are: lane 2, 25 pg pCMC1204 plus 5 μg of Coker 312 DNA; lane 3, 12.5 pg pCMC1204 plus 5 μg Coker 312, DNA; lane 4, 5 μg Coker 312, DNA; lane 5, 5 μg DNA from plant no. 1427; lane 6–16, 5 μg DNA from self-pollinated progeny. Positions and sizes of molecular length markers (*Hind* III digest of bacteriophage lambda DNA) are indicated on the left. Lanes 2 and 3 correspond to approximately 1 and 0.5 copies of pCMC1204 per cotton genome, respectively, based on a genome size of 3.2×10^9 base pairs (28).

ACKNOWLEDGMENTS

The authors wish to thank J. Burkholder, H. Poser, S. Downing, and E. Pahuski for technical assistance; the greenhouse staff, L. Hummel, S. Steinmann, R. Larson, and K. Busjahn for care of the transgenic plants; and W.J. Brill for critical review of the manuscript.

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