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Inheritance and structure of foreign DNA in progenies of transgenic tobacco obtained by direct gene transfer

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Summary. One of the transformed tobacco plants obtained by direct DNA transformation possessed two marker genes, a chimeric aminoglycoside phosphotransferase and nopaline synthase genes. Selfed progenies of this plant (T3-d) showed stable inheritance of these two genes. The minimum size of foreign DNA integrated into tobacco genome was estimated to be 5.4 kbp. A deleted nopaline synthase gene co-existed with an intact gene. The linkage analysis indicated that two transformants, T1-b and T3-c, possessed foreign DNA inserted in different chromosomes or in different sites of the same chromosome that recombine freely.

Key words: Tobacco – Direct DNA transformation – Insertion site – Linkage analysis

Introduction

Recent advances in DNA-directed transformation studies made it possible to transfer foreign genetic materials in the form of plasmid DNA into plant protoplasts (Paszkowski et al. 1984; Deshayes et al. 1985; Hain et al. 1985). Intensive studies concerning the genetic analysis of foreign DNA were carried out in *Nicotina* (Potrykus et al. 1985). Previously, we reported the successful introduction of bacterial plasmids containing two marker genes, i.e. the kanamycin resistant and nopaline synthase gene into tobacco protoplasts (Uchimiya et al. 1986). Among kanamycin resistant transformants, several clones capable of expressing both enzymes were identified. Thus, we selected one of these clones for molecular analysis of the fate of inserted genes. In this communication, the inheritance and structure of inserted plasmid DNA in the progenies of transformants is described. Linkage analysis of inserted genes using two different transformants is also presented.

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Materials and methods

Plant materials

Previously, we obtained 17 transformants from mesophyll protoplasts of tobacco (*Nicotiana tabacum* cv 'Petit Havana' SR1) treated with the vector pCT1T3 (Uchimiya et al. 1986). We were able to obtain seeds from 12 of them. We selected one clone (T3-d) for the present analysis. Two other clones, T1-b and T3-c, were used for a linkage study.

APH(3')II assay

Aminoglycoside phosphotransferase was analyzed according to Schreier et al. (1985).

Nopaline detection

Leaves (approximately 10 mg) were squashed and spotted onto Whatman paper for electrophoresis after Otten and Schilperoort (1978).

DNA analysis

DNA was prepared from young leaves (10 g F.W.) of transformed plants (Paszkowski et al. 1984). Extracted DNA was purified by CsCl density gradient centrifugation. Ten microgram DNA digested with restriction enzyme(s) was subjected to agarose gel electrophoresis followed by blotting onto a nitrocellulose filter and hybridized with multiprime-translated probe DNA (Southern 1975).

Results and discussion

Genotype and DNA analysis

Our previous work (Uchimiya et al. 1986) proved that 3 out of 17 clones expressing kanamycin resistance also

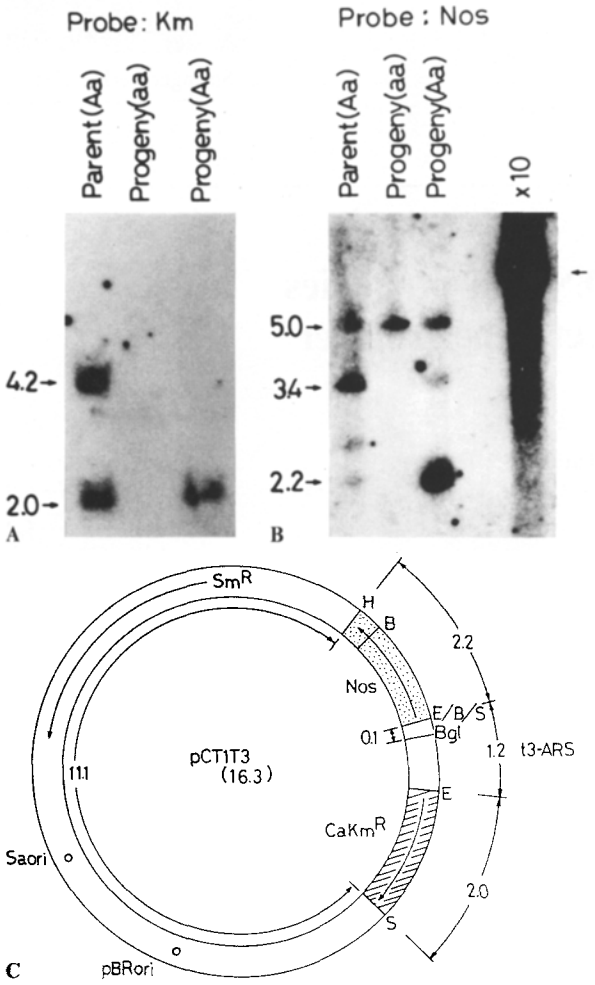

Fig. 1. **A** Autoradiograms of Southern blot hybridization of DNA prepared from a transformed plant (parent *Aa*) and its two progenies, no. 10 (*aa*) and no. 11 (*Aa*). DNA digested with EcoRI and SalI was applied to each track. The EcoRI-BglII fragment of pH1K1 (Uchimiya et al. 1986) was used as a probe for kanamycin resistant gene (Km). **B** DNA digested with EcoRI and HindIII was applied to each track. The EcoRI-HindIII fragment of pUC12H23ADIII (Uchimiya et al. 1986) was used as a probe for nopaline synthase gene (Nos) and for the HindIII digest of pUC12H23 (6.1 kbp) was served for a reconstruction of copy number (×10). *Numerals* indicate kbp. **C** Physical map of pCT1T3. NOS: Nopaline synthase gene; $CaKm^R$: A chimeric gene consisting of promoter and terminator of Cauliflower mosaic virus gene VI, the aminoglycoside phosphotransferase structural gene from Tn5; Sm^R: Streptomycin resistant gene; t3-ARS: 1.2 kbp autonomously replicating sequences in yeast isolated from tobacco chromosomal DNA (Uchimiya et al. 1983); B: BamHI; Bg: BglII; E: EcoRI; H: HindIII; S: SalI. *Numerals* indicate kbp. *Shaded box:* sequences homologous to kanamycin resistant gene. *Dotted box:* sequences homologous to nopaline synthase gene

produced nopaline. We have chosen a subclone T3-d for the present analysis. Seeds from selfing T3-d segregated green (resistance) and white (sensitive) seedlings in a 3:1 ratio on the medium containing kanamycin sulfate (200–400 μg/ml). This result indicates that foreign DNA

Table 1. Genotype of progenies obtained from selfing T3-d. Seeds were germinated on medium containing 200–400 μg/ml kanamycin sulfate. After 1–2 weeks, the number of seedlings with green (resistant: R) or white (sensitive: S) leaves were counted. Probability (*P*) at the 95% level was obtained from χ^2 test

Individual no.	No. plants			Expected ratio	χ^2	*P*	Genotype determined
	Total	R	S				
1	76	55	21	3:1	0.281	0.5–0.6	*Aa*
2	58	42	13	3:1	0.055	0.8–0.9	*Aa*
3	65	63	0	1:0	0.0	1.0	*AA*
4	63	58	0	1:0	0.0	1.0	*AA*
5	64	60	0	1:0	0.0	1.0	*AA*
7	110	73	30	3:1	0.935	0.7–0.8	*Aa*
8	65	63	0	1:0	0.0	1.0	*AA*
10	83	0	75	0:1	0.0	1.0	*aa*
11	163	104	44	3:1	1.766	0.1–0.2	*Aa*
12	74	72	0	1:0	0.0	1.0	*AA*
13	105	104	0	1:0	0.0	1.0	*AA*
14	90	71	9	3:1	8.517	0.0–0.5	*Aa*

was inserted in one of a set of tobacco chromosomes. For further analysis of the inheritance of inserted genes, 12 plants were randomly selected from the selfed progeny of T3-d. Furthermore, each plant was selfed and seeds obtained were germinated on kanamycin containing medium. From the results (Table 1), genotype of each plant as to kanamycin resistance was determined; number of the resistant homozygote (*AA*), heterozygote (*Aa*) and sensitive homozygote (*aa*) was 6, 5 and 1, respectively. Fig. 1 shows that foreign DNA sequence containing kanamycin resistant gene (Km) was present in the transformant and its progeny. A parent T3-d (genotype: *Aa*) possessed two bands, 2.0 and 4.2 kbp in size. The 2.0 kbp fragment was also seen in progeny no. 11 (genotype *Aa*). In the case of nopaline synthase gene (Nos), the 2.2 kbp fragment was detected in the T3-d (*Aa*) and progeny no. 11 (*Aa*). The 5.0 kbp fragment was also found in plant no. 10 (*aa*), which does not produce nopaline. The 3.4 kbp fragment seen in the parent could be a partial digest of the inserted DNA.

In order to determine the precise structure of integrated DNA, a resistant, homozygous, T3-d-12 (*AA*) was analyzed (Fig. 2). The 7.8 and 13 kbp fragments from EcoRI and HindIII digestion, respectively, hybridized to the Km probe. With the Nos probe, 6.6 and 8.1 kbp EcoRI fragments and 5.4 and 13 kbp HindIII fragments were formed. Double digestion with EcoRI and HindIII produced 2.2 (major), 4.7 (major) and 3.5 (minor) kbp fragments. These results indicate that two marker genes were located on the same chromosome. Since nopaline was not produced in progeny no. 10 (*aa*), a deleted nos gene could also be inserted. We also found a portion of plasmid DNA (~7 kbp) in rice cells transformed with a

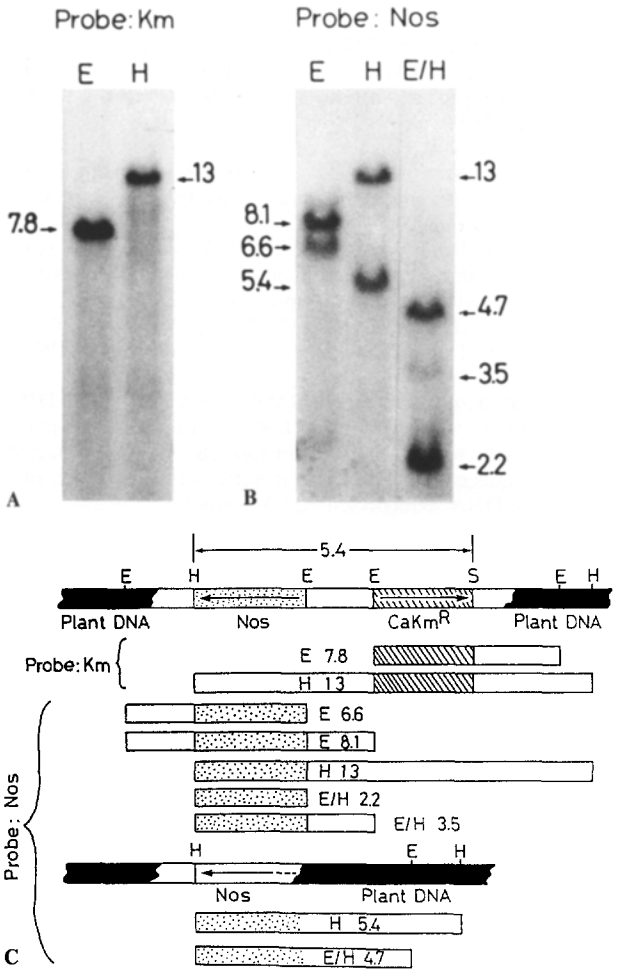

Fig. 2A–C. Autoradiograms of Southern blot hybridization of DNA prepared from a resistant, homozygous progeny (*AA*) of T3-d; expected integration pattern of foreign DNA into the host genome is presented in the bottom; each description can be seen in the legend for Fig 1

similar plasmid, pCT2T3 (18.3 kbp in size) (Morota and Uchimiya 1987). Using a rather small plasmid, pABD1 (5.3 kbp in size), Potrykus et al. (1985) reported the integration of a functional gene with several non-functional copies and plasmid sequencies in tobacco chromosomal DNA. Structural change and concatemerization of plasmid DNA were also noted in some cases, regardless of different DNA transfer methods such as calcium phosphate coprecipitation (Czernilofski et al. 1986) and electroporation (Riggs and Bates 1986). These results suggest that alteration of plasmid molecules may occur in the process of DNA integration into host DNA.

Cross experiments to determine site of integration in different transformants

In *Nicotiana* species, determination of the precise location of foreign gene insertion is not possible due to the lack of appropriate marker genes for individual chromosomes. We tried to determine whether the inserted gene was located on the same chromosome in different transformants. If a foreign gene is integrated in an identical site of the same chromosome, the F_1 population of the cross between two transformants (both supposed to be heterozygous) should segregate resistant (R) and sensitive (S) plants into a 3:1 ratio. Self-pollination of a resistant homozygote or its testcross to a sensitive homozygote should result in an uniform resistant progeny. Selfing or testcrossing of a heterozygote should give a 3:1 or 1:1 segregation ratio, respectively. If foreign gene is integrated in the different sites of the same chromosome, or in different chromosomes, a cross between two such transformants will segregate two- and one-gene resistant and sensitive plants in a 1:2:1 ratio, the R:S ratio being 3:1. When the two-gene resistant plant is selfed or testcrossed to the sensitive homozygote, the R and S type plants will segregate in a $1-\frac{p^2}{4}:\frac{p^2}{4}$ or $1-\frac{p}{2}:\frac{p}{2}$ ratio, respectively, where p is the crossover value between the two sites. If they locate far apart on the same chromosome or on different chromosomes, undergoing free recombination, these ratios become 15:1 and 3:1, respectively. Selfing or testcrossing of the one-gene resistant plant gives 3:1 or 1:1 segregation, respectively. We crossed two different transformants, T1-b and T3-c

Table 2. Genetic analysis of F1 hybrids between two transformed lines, T1-b and T3-c. For details see Table 1

Individual no.	No. plants			Expected ratio	χ^2	*P*
	Total	R	S			
1 (self)	77	54	23	3:1	0.974	0.3–0.4
1 × SR1	105	55	45	1:1	1.00	0.3–0.4
2 (self)	89	81	6	15:1	1.087	0.2–0.3
2 × SR1	77	58	19	3:1	0.004	0.9–1.0
3 (self)	115	79	33	3:1	1.19	0.2–0.3
3 × SR1	78	44	32	1:1	1.895	0.1–0.2
4 (self)	102	91	8	15:1	0.566	0.4–0.5
4 × SR1	95	81	13	3:1	6.255	0.0–0.1
5 (self)	112	108	4	15:1	1.371	0.2–0.3
5 × SR1	75	57	18	3:1	0.04	0.8–0.9
6 (self)	110	82	28	3:1	0.012	0.9–1.0
6 × SR1	82	40	41	1:1	0.012	0.9–1.0
7 (self)	88	83	5	15:1	0.048	0.8–0.9
7 × SR1	91	70	20	3:1	0.370	0.5–0.6
8 (self)	82	77	3	15:1	0.853	0.3–0.4
8 × SR1	87	58	17	3:1	0.217	0.6–0.7
9 (self)	53	38	15	3:1	0.308	0.5–0.6
9 × SR1	81	40	40	1:1	0.0	1.0
10 (self)	115	85	29	3:1	0.012	0.9–1.0
10 × SR1	142	74	67	1:1	0.347	0.8–0.9

(Uchimiya et al. 1986), both containing one copy of an intact kanamycin resistant gene (unpublished data). Individual F_1 plants were selfed or testcrossed with SR1 as a pollen donor, and seeds produced were tested for kanamycin resistance. As summarized in Table 2, five plants showed 15:1 segregation when selfed. Testcross resulted in either 1:1 or 3:1 segregation. Therefore, two transformed lines, T1-b and T3-c, contained the foreign gene in different chromosomes or on different sites of the same chromosome that recombine freely. Peerbolte et al. (1985) reported the integration of foreign DNA in three different chromosomes based on the segregation of marker DNAs in tobacco transformants. Using the in situ hybridization technique, Mouras et al. (1987) found the localization of transferred DNA in different tobacco chromosomes in single blocks. These observations, together with the results of this study, provide convincing evidence for random foreign DNA insertion into plant chromosomes.

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