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Molecular tagging of the tobacco chromosome carrying the TMV-resistance gene (N gene) by *Agrobacterium*-mediated transformation

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Summary. The hypersensitive response of tobacco to inoculation with tobacco mosaic virus (TMV) is controlled by a single dominant gene, the *N* gene. As a first step in localizing and transferring the *N* gene, we have prepared a line of tobacco plants in which the kanamycin-resistance (Km^r) gene is closely linked to the *N* gene. *Nicotiana tabacum* plants heterozygous for the *N* gene were transformed to Km^r by *Agrobacterium* carrying pMON200. Eighty-nine independent transformed clones were regenerated and were backcrossed with nontransformed, TMV-sensitive plants. Progeny from these crosses were screened first for Km^r; then the Km^r progeny were inoculated with TMV and scored for the hypersensitive response. Of the initial 89 clones, 68 appeared to have integrated a single functional Km^r gene. Initial tests for TMV resistance indicated possible linkage between Km^r and the *N* gene in 11 plants. With further testing, linkage has been established for two of these plant lines. In one of these lines, the two genes were 30–40 map units apart, and evidence of somatic instability in the linkage was obtained. However, in the second line, linkage between Km^r and the *N* gene was tight, and recombination between the genes in this case was only 5%. Southern hybridization revealed that this plant contained only a single copy of the Km^r gene. Linkage between Km^r and the *N* gene in this plant line has been verified in each of two additional backcross generations.

Key words: Tobacco mosaic virus – *Nicotiana tabacum* – Kanamycin resistance – Hypersensitive response

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Abbreviations: nptII, Neomycin phosphotransferase gene; Km^r, kanamycin resistant; Km^s, kanamycin sensitive; TMV, tobacco mosaic virus; TMV-R, TMV resistant; TMV-S, TMV sensitive

Introduction

Tobacco mosaic virus (TMV) is a serious pathogen of tobacco (Gooding 1986), and closely related strains, such as the tomato mosaic virus, are important pathogens in other solanaceous plants (Broadbent 1976). In tobacco, resistance to infection by TMV is afforded by a highly effective hypersensitive response, which is under the control of a single dominant gene, the *N* gene. The *N* gene originated in *Nicotiana glutinosa* and was transferred to *N. tabacum* through an interspecific cross (Clausen and Goodspeed 1925). Extensive backcrossing with *N. tabacum* and selfing indicated that the *N* gene behaves as a classical dominant monogenic trait (Holmes 1938). Cytological analyses of these TMV-resistant (TMV-R) tobaccos suggested that the entire *N. tabacum* H chromosome has been replaced by the analogous chromosome of *N. glutinosa* (Gerstel 1943, 1945). The molecular mechanism by which the *N* gene triggers a hypersensitive response to inoculation with TMV is not known, and the lack of proven isogenic lines has hampered efforts to characterize the action of this gene. Efforts to clone the *N* gene have been unsuccessful thus far (Dunigan et al. 1987; Hehl and Baker 1991).

In this paper, we describe the introduction of a neomycin phosphotransferase gene construction (*nptII* gene or kanamycin-resistance gene) at a site closely linked to the *N* gene in *N. tabacum*. The strategy was to transform plants heterozygous for the *N* gene to kanamycin resistance by *Agrobacterium*-mediated transformation and then to select for transformants in which the *N* gene and the Km^r gene were linked. The approach is based on the assumption that T-DNA integration occurs at random genomic sites. Our goal in creating a tobacco line with linkage between the kanamycin-resistance (Km^r) gene and the *N* gene is to use protoplast

fusion to transfer the *N* gene to other plant species. However, the tobacco line we obtained, in which the *N* gene and the Km^r gene are tightly linked, may also become useful for mapping and cloning the *N* gene.

Methods

Transformation and kanamycin-resistance tests

Plants of *N. tabacum* var 'Xanthi', genotypes *NN* and *nn*, were obtained from Dr. Verne Sisson, USDA Tobacco Research Laboratory, P.O. Box 1555, Oxford, N.C., USA. Tobacco plants heterozygous for the *N* gene (genotype *Nn*) were prepared by crossing these two 'Xanthi' cultivars, and transformation to kanamycin resistance was by the leaf-disc transformation procedure (Rogers et al. 1986) using *Agrobacterium tumefaciens* carrying PTiB6S3-SE with pMON200 cointegrated (Rogers et al. 1986; the Agrobacterium strain was the generous gift of Dr. Stephen Rogers, Monsanto). *Agrobacterium*-treated leaf discs were cultured on solidified RMB medium (Bates 1990) supplemented with 0.5 mg/l carbenicillin plus 0.3 mg/l kanamycin. Independent, transformed clones were identified as discrete regenerating sectors on the leaf discs and were transferred to individual petri dishes for plant regeneration. Regenerated shoots were rooted on P medium (Bates 1990) supplemented with 0.1 mg/l kanamycin.

Kanamycin-resistant plants were transferred to the greenhouse, grown to maturity, and backcrossed with untransformed 'Xanthi' plants of genotype *nn*. Inheritance of Km^r was scored by counting the number of surface-sterilized seeds that germinated on solidified P medium supplemented with 0.1 mg/l kanamycin, at 27 °C, 20 $\mu E/m^2 s$ fluorescent illumination. After 3–4 weeks the proportion of Km^r to kanamycin-sensitive (Km^s) seedlings was determined. Under these conditions the Km^s seedlings germinate and remain green but do not develop true leaves or roots, whereas the Km^r seedlings grow normally. The Km^r seedlings were transferred to soil. Flats of the seedlings were left covered with clear plastic wrap for 1–2 weeks to allow the plants to harden.

TMV inoculations

After 6–8 weeks in soil, leaves of the Km^r seedlings were rubbed with a suspension of TMV particles in a slurry of 0.1 *M* K-phosphate buffer (pH 7) and carborundum powder (400 mesh, 10% w/v). Cotton-tipped applicators were used for these inoculations. Five days after inoculation, the treated leaves were scored for the local lesions that characterize the hypersensitive response of TMV-resistant (TMV-R) tobacco plants. Two strains of TMV were used for the inoculations. The initial screening of the seedlings for TMV resistance was done with an uncharacterized TMV strain obtained from Dr. Guy Gooding (Department of Plant Pathology, North Carolina State University). Once a plant line expressing both TMV-R and Km^r was identified, linkage was verified by the inoculation of Km^r seedlings with the U1 strain of TMV.

DNA isolation and Southern blotting

DNA was isolated from young leaves by the procedure of Dellaporta et al. (1985). The plant DNA was then RNase treated and extracted with phenol-chloroform. The plasmid (pMON200 in *E. coli*) was isolated by standard alkaline lysis procedures (Maniatis et al. 1982). DNA concentrations were determined by the diphenylamine assay (Thompson and Dvorak 1989). Plant and plasmid DNA's were digested with restriction enzymes according to the manufacturer's instructions and were separated by electrophoresis on 1% agarose gels. Southern transfers and hybridizations were performed as described by Maniatis et al. (1982). Radiolabeled probes were prepared by random primer labeling (Feinberg and Vogelstein 1983) of a 1.3-kb *Eco*RI/*Bam*HI fragment of pMON200. This fragment contains only the coding region of the *nptII* gene. Hybridization was visualized by autoradiography using Kodak XAR-5 film and was quantified by direct counting of radioactivity in individual bands on the nitrocellulose with a Betascope Model 603 Blot Analyzer (Betagen Corp, Waltham, Mass.).

Results

Initial screening for linkage

Plants were regenerated from 89 independent transformed lines, and these R_0 plants were backcrossed with Km^s, TMV-sensitive (TMV-S) tobacco. Because transformants having T-DNA integrated at a single locus would be the easiest to analyze for linkage, the inheritance of Km^r was scored to identify plants carrying a single T-DNA insert. Of the original 89 plants, 68 gave approximately 1 : 1 ratios of $Km^r : Km^s$ progeny, which is consistent with the integration of a functional *nptII* gene at a single locus. Twelve plants had two copies integrated at different sites, and four plants had three copies integrated. The remaining five plants yielded progeny that were either all Km^s or had very weak kanamycin resistance.

The $Km^r : Km^s$ inheritance ratios for the 68 candidate lines are shown in Table 1. Because the purpose of this study was to identify transformants in which Km^r and TMV-R were linked, the pattern of inheritance of Km^r was not analyzed in depth in this initial screen, and on the basis of chi-squared tests, several plants had $Km^r : Km^s$ ratios that did not fit a clear pattern of Mendelian inheritance. These unusual inheritance ratios are marked by asterisks in Table 1. Some of them may be explained by the small number of progeny scored, but others appear to reflect genuine deviations. Such deviations might arise by T-DNA integration at a locus affecting gamete or embryo fitness. Occasional inactivation of the *nptII* gene could also result in skewed ratios.

To determine which of the 68 candidate lines having single integrated *nptII* genes also exhibited linkage between the *nptII* gene and the *N* gene, we scored their Km^r F_1 progeny for a hypersensitive response to TMV inoculation. Because of the large amount of hand labor involved, only small numbers of Km^r progeny were tested in the initial screen. The strategy was to use this initial screen to identify plants in which the two genes might be linked and then to test larger numbers of the progeny of just those plants. The results of the initial screen for linkage are shown in Table 1. Any plant whose Km^r progeny displayed a significant excess of either TMV-R or TMV-S progeny was a candidate for retesting. These

Table 1. Segregation of kanamycin resistance and hypersensitivity among transformants that carry a single functional copy of the *ntpII* gene. R_0 transformants were backcrossed with Km^s/TMV-S tobacco, and the progeny were scored for Km^r. The km^r progeny were transferred to soil, grown to large size, inoculated with TMV, and scored for a hypersensitivity

Plant	Km^r/Km^s	Km^r seedlings
		TMV-R/TMV-S
1	92/84	57/73
2	90/91	58/45
3	184/220	77/47**
4	148/100*	38/64**
5	164/162	93/87
6	71/80	57/55
7	104/102	90/66**
8	197/226	ND
9	100/101	68/92
10	91/97	58/67
11	253/215	53/85**
12	104/103	75/91
13	270/277	88/84
14	120/123	91/77
15	226/249	93/92
16	359/291*	83/94
17	86/82	90/86
18	251/305*	72/89
19	208/219	92/87
20	214/210	77/96
21	281/266	100/83
22	199/190	11/135**
23	136/118	39/33
24	218/288	106/80**
25	142/127	127/108
26	132/144	68/81
27	46/45	54/79
28	217/202	82/87
29	122/107	89/98
30	92/98	67/60
31	184/162	83/81
32	91/89	88/85
33	266/291	83/76
34	228/208	88/91
35	104/99	73/103**
36	57/48	27/39
37	89/83	87/97
38	388/432	89/81
39	275/267	82/91
40	50/51	72/74
41	268/333*	76/96
42	147/160	48/50
43	223/192	78/82
44	317/378*	73/99**
45	55/44	36/65**
46	91/122*	37/42
47	100/101	58/99**
48	89/91	56/48
49	53/56	119/122
50	169/177	88/92
51	75/78	67/82
52	87/89	33/44
53	55/59	72/62
54	177/150	99/113
55	309/354	79/83
56	51/55	69/60
57	79/52*	93/87
58	82/85	74/66
59	61/52	86/86
60	250/299*	75/85
61	248/256	98/93
62	53/58	82/107
63	67/66	85/87
64	62/40*	67/74
65	34/32	96/81
66	89/83	85/93
67	105/100	135/104**
68	174/160	76/91

* Indicates plants in which there is statistical evidence ($P<0.01$) of deviation from a 1:1 ratio of Km^r:Km^s progeny
** Indicates plants in which there is a deviation ($P<0.01$) from a 1:1 ratio of TMV-R:TMV-S individual among the Km^r progeny

Table 2. Transformed plants for which evidence of linkage between Km^r and TMV-R was obtained when large numbers of seedlings were tested. Only the segregation of TMV resistance among the Km^r progeny of these plants is shown in this table

Plant	Km^r seedlings	Map units apart
	TMV-R/TMV-S	
3	354/152 ($P<0.001$)	30
22	30/601 ($P<0.001$)	5

candidate plants are marked by the double asterisks in Table 1.

Follow-up tests for linkage

For the 11 plants shown in Table 1 in which preliminary evidence indicated linkage between TMV-R and Km^r, larger numbers of Km^r seedlings were inoculated with TMV in a preliminary effort to verify linkage. After this retesting, 2 plants remained for which evidence of linkage was still strong. The expanded data set for these plants is shown in Table 2.

In plant 3, the chi-squared test for linkage was highly significant for seeds of the first two capsules collected, but the genes were not closely linked. After these data were obtained, plant 3 was backcrossed again with Km^s/TMV-S tobacco, and seeds from six additional capsules were scored for the inheritance of Km^r and TMV-R. The inheritance of Km^r among seeds from these capsules still showed statistically significant linkage between Km^r and TMV-R (261 Km^r/TMV-R seedlings to 218 Km^r/TMV-S

seedlings; $P=0.05$), but the map distance between the genes was now much greater (45 cM). The inheritance of Km^r in these capsules was normal (1:1 ratio of Km^r to Km^s seedlings). The basis of this pattern of inheritance was not studied further in this plants, but it may reflect somatic recombination between Km^r and TMV-R when these two genes are distantly linked.

Plant 22 was of particular interest because the TMV inoculations indicated a highly skewed inheritance of the *N* gene among the Km^r progeny. Testing of larger numbers of seedlings verified this pattern of inheritance (Table 2); similar results were obtained when additional backcross capsules were tested. When seeds obtained from the backcrossing of plant 22 were tested for TMV-R, without first being tested for Km^r, a 1:1 ratio of TMV-R:TMV-S progeny was obtained (data not shown). This result indicates that TMV-R is inherited in a Mendelian fashion as a single dominant gene in this plant. Kanamycin-resistance was also inherited in a Mendelian fashion in this plant (see Table 1).

The most likely explanation for the inheritance seen in plant 22 is the integration of T-DNA into the homolog carrying the recessive allele of the *N* gene at a site about 5 map units from the *N*-gene locus. If this explanation is correct, then the small number of Km^r/TMV-R progeny recovered in these crosses must have resulted from crossing-over between these genes. Thus, backcrossing these Km^r/TMV-R F_1 progeny should result in a large excess of Km^r/TMV-R F_2 progeny. As shown in Table 3, this is precisely what was observed. In this experiment, three separate Km^r/TMV-R F_1 progeny of plant 22 were both selfed and backcrossed. In the backcrosses, a 1:1 inheritance of Km^r:Km^s was obtained, and, as predicted, about 95% of the Km^r F_2 progeny were also TMV-R. The selfed progeny displayed a 3:1 inheritance of Km^r, and all of the Km^r progeny tested gave hypersensitive responses when inoculated with TMV.

To stabilize linkage between Km^r and TMV-R, Km^r/TMV-R F_2 progeny obtained from the selfing of plants 22-1 and 22-2 were again selfed and backcrossed. From these crosses two plants have been identified that breed true for Km^r and TMV-R.

Plant 22-1 carries a single integrated copy of the nptII gene

Use of this transformed plant line for further mapping studies of the *N* gene or for experiments seeking to transfer the *N* gene to other species by somatic hybridization will be facilitated by a molecular genetic analysis of the T-DNA insert in these plants. It is particularly important to learn whether silent copies of the *nptII* gene are present at other loci in addition to the active *nptII* gene that has been followed in the crossing studies described above. Accordingly, genomic DNA from plant 22-1 was cut with several restriction enzymes and probed for the presence of the *nptII* gene (Fig. 1). The *Agrobacterium* strain used for transformation carried the plasmid pMON200 cointegrated (see Rogers et al. 1986 for a restriction map of this vector). Digestion of pMON200 DNA (lane 5) and genomic DNA isolated from plant 22-1 (lane 1) with *Eco*RI and *Bam*HI released the expected 1.3-kb fragment that hybridizes with the *nptII* gene probe. DNA from untransformed plants did not hybridize (data not shown). *Hin*dIII, *Bam*HI, and *Bgl*I each cut pMON200 only once – in the transformant; the second cut site will therefore be in plant genomic sequences. *Hin*dIII cuts pMON200 on the 5′ side of the *nptII* gene, whereas *Bam*HI and *Bgl*I cut on the 3′ side of this gene. For all three enzymes, digestion of 22-1 DNA yielded a single band of high molecular weight that hybridized to the *nptII* gene (Fig. 1, lanes 2–4). Because multiple integration sites should result in multiple bands on this Southern blot, these results are consistent with the presence of a single T-DNA insertion in 22-1. A copy number recon-

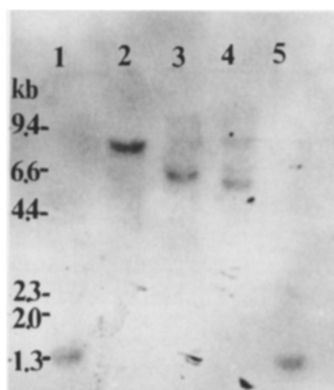

Fig. 1. Southern blot of integrated *nptII* gene in transformant displaying tight linkage between the *nptII* gene and the *N* gene. Genomic DNA was isolated from plant 22-1 (*lanes 1–4*) and plasmid pMON200 (*lane 5*). DNA's in *lanes 1* and *5* were cut with *Eco*RI and *Bam*HI. DNA's in *lanes 2, 3,* and *4* were digested with *Hin*dIII, *Bam*HI, and *Bgl*I, respectively. After transfer to nitrocellulose, the blot was probed with a ^{32}P-labeled fragment of the *nptII* gene

Table 3. Backcrosses verify linkage in plant 22. Three Km^r/TMV-R F_1 progeny of plant 22 were selfed and then backcrossed with Km^s Tmr-S tobacco. The resulting progeny were scored for the inheritance of Km^r and the Km^r F_2 progeny were further scored for TMV-R

Cross	Km^r/Km^s	Km^r progeny
		TMV-R TMV-S
22-1 backcrossed	101/86	189/5
22-2 backcrossed	107/137	190/12
22-3 backcrossed	53/50	228/8
22-1 selfed	111/35	10/0
22-2 selfed	71/26	57/0
22-3 selfed	139/45	53/0

struction was also performed by measurement of the radioactivity that hybridized to known amounts of 22-1 DNA and to pMON200 when these DNA's were probed with a radiolabeled fragment of the *nptII* gene. For pMON200, 9,370 counts of radioactivity hybridized to 33 pg of the plasmid, and 5,000 counts hybridized to 15 micrograms of 22-1 DNA. Given a genome size of 4.8 pg for a *N. tabacum* germ cell and a molecular size of 9.5 kg for pMON200, these results indicate the presence of only a single copy of the *nptII* gene per somatic nucleus of *N. tabacum*.

Discussion

Our data demonstrate the feasibility of using *Agrobacterium*-mediated transformation to introduce a molecular tag into a specific chromosome carrying a gene of interest. The recovery of large numbers of independent transformants having only single functional T-DNA integration sites facilitated the search for a plant with linkage between the *nptII* gene and the target gene, the *N* gene. We found that 78% of the transformants contained a functional *nptII* gene integrated at a single site. Approximately 16% of the transformants had functional inserts at two or more loci, and 6% yielded progeny displaying attenuated or no expression of Km^r. These data are in agreement with previous studies in which the number of T-DNA integration sites has been examined in a large number of transformants (Budar et al. 1986; Delores and Gardner 1988; Heberle-Bors et al. 1988).

The probability of introducing the *nptII* gene into the chromosome carrying the *N* gene depends on the number of chromosome pairs, which is 24 for *N. tabacum*. On the basis of a binomial distribution and the assumption that all the chromosomes are of equal size, it can be calculated that among 68 transformants the chance of obtaining no transformants in which the *nptII* gene has integrated into the *N*-gene-carrying chromosome is only 5% and the chance of obtaining one or two transformants with the two genes located on the same chromosome is 40%. For species with fewer chromosomes, even smaller numbers of transformants would be needed for successful chromosome tagging. This calculation assumes that T-DNA integration is random. Because we were not able to map T-DNA integration to specific chromosomes in all our transformants, we cannot directly verify this assumption, but Wallroth et al. (1986) reported a linkage analysis for nine independent *Petunia* transformants. They found that the T-DNA integration sites were different in all nine transformants and that four of the seven pairs of *Petunia* chromosomes had T-DNA insertions. Although the sample size is small, these data are consistent with random T-DNA integration. Chyi et al. (1986) have reported a similar linkage analysis of transformed tomatoes, and their data also indicate random chromosomal integration of T-DNA.

Genome size presents another complexity when the chance for success in chromosome tagging is estimated. Plants with large genomes are likely to have chromosomes with map lengths greater than 100 cM. In this case crossing-over becomes so frequent that the two ends of the chromosome act as independent linkage groups. The recombination length of tobacco chromosomes is not known, but it is likely that many are more than 100 cM long. For example, in tomato, which has a much smaller genome than tobacco, 6 of the 12 chromosomes are 100 cM or longer (Zamir and Tanksley 1988). The introduction of the *nptII* gene into a site closely linked to a specific endogenous gene, and not just into a specific chromosome, will depend even more strongly on genome size. Although we argue that our recovery of transformants in which the *nptII* gene and the *N* gene are linked was a statistically likely event, the recovery of one in which the two genes are only 5 cm apart is undoubtedly fortuitous. However, in plants with smaller genomes than tobacco, it might not be unreasonable to expect to obtain close linkage. Indeed, in *Arabidopsis*, *Agrobacterium*-mediated transformation has been used for gene cloning through the insertional inactivation caused by T-DNA integration into active genes (Feldmann and Marks 1987; Yanofsky et al. 1990).

The tobacco line described here could be used in several ways for the localization and eventual cloning of the *N* gene. In particular, the presence of T-DNA sequences near the *N* gene could serve as a starting point for chromosome walking or for the isolation of RFLP's more closely linked to the *N* gene, which could in turn be used for the initiation of chromosome walking.

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