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ORIGINAL PAPER

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Transfer of resistance to potato virus Y (PVY) from *Nicotiana africana* to *Nicotiana tabacum*: possible influence of tissue culture on the rate of introgression

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Abstract A disomic chromosome addition line of tobacco, *Nicotiana tabacum* L., was established previously that possesses a single chromosome pair from *N. africana* [Merxm. and Buttler]. This addition chromosome carries a gene that confers increased resistance to severe strains of potato virus Y (PVY). Methods to increase the probability of gene transfer from alien chromosomes to tobacco ($2n=48$) are desired. In the research described here, the PVY resistance gene was transferred to a tobacco chromosome from the *N. africana* addition chromosome in seven independent cases. One introgression event was obtained using conventional backcrossing of the disomic addition line to *N. tabacum* cv. Petite Havana, while the remaining six events were obtained using a scheme that involved exposure of explants of the addition line to tissue culture. Twenty-six derived $2n=48$ individuals heterozygous for PVY resistance were found to exhibit 24 bivalents or 23 bivalents + 2 univalents at metaphase I. Ovular transmission rates for the PVY resistance factor ranged from 25% to 52%, while pollen transmission rates were much lower, ranging from 0 to 39%. Fifty-one random amplified polymorphic DNA (RAPD) markers specific for the intact addition chromosome were identified and used to characterize derived $2n=48$/PVY-resistant genotypes. Variability was observed among these plants with respect to the total number of *N. africana* RAPD markers that were present, which is an indication that crossing over was occurring within each of the seven introgressed chromosome segments. A limited molecular marker-assisted backcrossing experiment allowed for selection of a $2n=48$/PVY-resistant individual that possessed only 6 of the 51 original *N. africana* RAPD markers. In vitro culture is potentially a valuable system for increasing the rate of alien gene transfer in tobacco, and the successful transfer of PVY resistance from *N. africana* may allow for an increased level and range of resistance to this virus in tobacco.

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Introduction

Cultivated tobacco, *Nicotiana tabacum* L. ($2n=48$), is susceptible to crop loss caused by potato virus Y (PVY), a pathogen of great economic importance to the tobacco industry worldwide (Lucas 1975; Delon et al. 1993). Resistance to many isolates is conferred by the irradiation-induced recessive gene *va* from cv. Virgin A Mutante (Koelle 1961) and by its allelic forms found in several other cultivars (Wernsman 1992a). While partial resistance has also been found in gametoclonal variant NC602 (Witherspoon et al. 1991), no one source provides complete resistance to all strains of PVY, and additional sources of resistance would be valuable for increasing the range and level of resistance in *N. tabacum*.

Lucas et al. (1980) found an accession of *N. africana* [Merxm. and Buttler] ($2n=46$) to be immune to three strains of PVY, and Wernsman (1992b) subsequently developed a chromosome addition line, NC152 ($2n=50$), which possesses a pair of homologous chromosomes from *N. africana* . The addition line does not exhibit the extreme levels of PVY resistance of *N. africana* per se, but it does carry a gene(s) that provides resistance to the necrotic effects of severe strains of PVY (Campbell et al. 1994). NC152 may be resistant to some *va* -breaking isolates and possesses tolerance to several isolates of tobacco etch virus (unpublished observations). Resistance acts in an additive nature as the disomic addition line exhibits less stunting than the monosomic addition line.

The usual objective in introgression is to transfer a gene of interest from an alien species to a chromosome

of the cultivated species' genome with minimal amounts of accompanying foreign chromatin. This is often complicated by strong preferential bivalent pairing between the chromosomes of the recipient species' genome and low levels of homology between the donor and recipient chromosomes. Gerstel et al. (1979) reported chromosome pairing in *N. tabacum* × *N. africana* hybrids to be of the "low-variable" type. Efforts by Witherspoon (1987) were not successful in isolating genotypes in which the *N. africana* addition chromosome of NC152 had recombined with a chromosome of the *N. tabacum* genome.

There is a strong need to develop methods that will facilitate alien gene transfer when the affinity for chromosome pairing is low. Strategies for effecting recombination with addition or substitution chromosomes in wheat have included the use of pollen or seed irradiation, mutations affecting homoeologous chromosome pairing, and whole-arm substitution brought about by misdivision of univalent chromosomes during meiosis (Riley et al. 1968; Sears 1981, 1993; Knott 1987; Friebe et al. 1996). In vitro culture frequently induces chromosome breakage and fusion in plant species (Lee and Phillips 1988; Kaeppler et al. 1998, 2000). Translocations were found to be the most frequent chromosomal abnormality in maize and oat plants regenerated from tissue culture (Benzion et al. 1986; Kaeppler et al. 1998). Such observations led Larkin and Snowcroft (1981) and Larkin et al. (1989) to propose in vitro culture as a possible means for introgressing desirable alien genes into the genomes of a cultivated crop species.

The first objective of this investigation was to determine the value of tissue culture relative to simple backcrossing as a means of transferring genetic material from the *N. africana* chromosome of NC152 to the *N. tabacum* genome. The goal was to identify multiple independent events in which the PVY resistance gene was transferred to a *N. tabacum* chromosome. Campbell et al. (1994) previously tagged the addition chromosome with a mutant *dhfr* transgene conferring resistance to the antibiotic methotrexate. Consequently, the determination of ovular *dhfr* transmission rates in BC_1F_1 families was used in an efficient preliminary screen to identify potential interchromosomal recombination events.

Tobacco breeding lines possessing disease resistance genes transferred from wild relatives have often had reduced commercial value, a consequence likely due to deleterious alien genes flanking the gene of interest (Chaplin et al. 1966; Legg et al. 1981; Johnson 1999; Linger et al. 2000). Young and Tanksley (1989) found the traditional backcross method to be very ineffective in reducing the size of an alien DNA segment flanking a disease resistance gene in tomato. This problem can be exacerbated when the donor chromosome is from a different species. The authors recommended the use of marker-assisted backcrossing to identify those individuals in which desirable recombination events had occurred. A second objective of this investigation was to identify a set of random amplified polymorphic DNA (RAPD) markers associated with the intact *N. africana* chromosome in NC152. This information was then used to determine the relative amounts of *N. africana* chromatin present in 48-chromosome PVY-resistant plants derived from multiple independent introgression events. Markers were used to determine the potential for crossing over within transferred chromosome segments and to select for reduced fragment sizes during backcrossing of the PVY resistance gene to an elite cultivar.

Materials and methods

Starting plant material and the generation of BC_1F_1 families

The PVY-resistant (PVY^R) disomic chromosome addition line NC152 ($2n=50$) was developed by adding a single pair of *N. africana* chromosomes to PVY-susceptible (PVY^S) tobacco cultivar McNair 944 (Wernsman 1992b). The addition chromosome was subsequently tagged with a mutant *dhfr* transgene that confers resistance to the antibiotic methotrexate (Mtx) to create line NC152-*dhfr* -996 (Campbell et al. 1994). BC_1F_1 families were derived from this line using two different schemes in order to determine the potential value of tissue culture for promoting recombination between the alien chromosome and the *N. tabacum* genome. Non-tissue culture-derived families were produced by first crossing 144 individual NC152-*dhfr* -996 plants with the $PVY^S/2n=48$ cv. Petite Havana. Individual F_1 plants were then backcrossed as females to Petite Havana to produce 144 independent BC_1F_1 families which, in most cases, were expected to segregate for the presence of the addition chromosome.

For the tissue culture regime, leaf discs from 12 different NC152-*dhfr* -996 plants were placed in tissue culture. Two hundred and sixty 1-cm^2 leaf discs were initially placed on solid MS culture medium (Murashige and Skoog 1962) for 2 days and then transferred to shoot regeneration medium comprised of MS inorganic salts supplemented with 4.0 mg l^{-1} indole acetic acid (IAA), 2.5 mg l^{-1} kinetin, 30 g l^{-1} sucrose, 250 mg l^{-1} cefotaxime, 0.5 mg l^{-1} Mtx, 100 mg l^{-1} kanamycin, and 7 g l^{-1} agar. The discs were transferred to fresh medium every 14–21 days. Regenerated shoots were removed periodically and transferred to rooting medium consisting of MS inorganic salts plus 30 g l^{-1} sucrose and 7 g l^{-1} agar. One hundred and forty-one rooted R_0 plants derived from 121 different leaf discs were selected and transferred to soil-filled pots in a growth room. Only one or two R_0 plants per explant were retained, thus ensuring a high number of independent regenerates. These regenerates were then crossed to Petite Havana. Individual F_1 plants were then backcrossed as females to Petite Havana to produce 141 BC_1F_1 families derived from tissue culture.

Identification of putative interchromosomal recombination events

The relative positions of *dhfr* and the PVY resistance factor on the addition chromosome were not known a priori. Due to the subtelomeric structure of the chromosome, however, it was predicted that they resided on the same chromosome arm. In the absence of interchromosomal recombination, it was expected that *dhfr* would be transmitted to BC_1F_1 individuals at a frequency of approximately 7–10% (Campbell et al. 1994; Lewis and Wernsman 2001). A much greater percentage of Mtx^R plants (close to 50%) was expected if a *N. africana* chromosome segment carrying *dhfr* had been transferred to a *N. tabacum* chromosome. The percentage of Mtx^R plants in each BC_1F_1 family was determined by germinating 121–847 surface-sterilized seeds per family on sterile medium consisting of MS inorganic salts (Murashige and Skoog 1962) supplemented with 1 mg l^{-1} Mtx and 7 g l^{-1} agar in 100×15-mm petri plates. A Chi-square test was used to determine if the frequency of putative introgression events obtained using the tissue culture scheme was significantly different from that observed for the non-tissue culture scheme.

It was hypothesized that *dhfr* had been transferred to a *N. tabacum* chromosome in families with 40–60% Mtx^R plants. Data were collected to determine if the PVY resistance gene was also transferred to a *N. tabacum* chromosome in these families. One hundred plants from each of these families were tested for resistance to both PVY and Mtx approximately 30 days after seeding (two true-leaf stage). Chi-square tests were used to determine if segregation differed significantly from 1:1 ratios.

The whole-plant response to Mtx was determined by placing three 0.70-cm-diameter surface-sterilized leaf discs per plant on solid MS culture medium supplemented with 4.0 mg l^{-1} IAA, 2.5 mg l^{-1} kinetin, 30 g l^{-1} sucrose, 7 g l^{-1} agar, and 1 mg l^{-1} Mtx. Approximately 14 days after plating, leaf discs were classified as Mtx^R (proliferation of shoots on medium) or Mtx^S (death of leaf disc).

PVY inoculation procedure

Plants were inoculated at the two true-leaf stage (approximately 30 days old) as described by Witherspoon et al. (1991) using a strain of PVY NN (NC isolate 78) maintained by our program. The nomenclature used to describe this strain reflects the necrotizing effects that it has on genotypes with or without the root-knot nematode resistance gene *Rk* (Gooding and Tolin 1973; Gooding and Lapp 1980). Plants were maintained in a growth room at 26 ± 3°C and were evaluated 11–15 days post-inoculation. PVY NN causes severe veinal necrosis 8 days after inoculation on genotypes possessing no genetic resistance. While variation for amounts of stunting, leaf crinkling, and mosaic was usually present among the inoculated plants, plants were classified as PVY^R or PVY^S according to whether or not veinal necrosis was present.

Identification of RAPD markers associated with the intact *N. africana* addition chromosome

A total of 1,216 random decamer primers (Operon Technologies, Alameda, Calif. and University of British Columbia, Vancouver, B.C., Canada) were screened for their ability to reveal RAPD polymorphisms between NC152-*dhfr* -996 and a bulk of three $2n=48/PVY^S$ tobacco lines (McNair 944, K326, and Petite Havana). DNA was isolated according to Johnson et al. (1995), except that a BIO 101 FastPrep machine (BIO 101, Holbrook, N.Y.) was used for tissue grinding. RAPD reactions were performed using a PTC-100 MJ Research Programmable Thermal Controller (MJ Research, Watertown, Mass.). Reactions were conducted in 22-µl volumes containing 15 ng genomic DNA, 10 m*M* Tris-HCl, pH 8.3, and 50 m*M* KCl (1× Stoffel buffer), 200 μM of each dNTP, 1.65 U Amplitaq DNA polymerase Stoffel fragment (Perkin Elmer, Foster City, Calif.), 4 m*M* $MgCl_2$, 22 µg bovine serum albumin, and 20 ng of primer. Amplifications were performed for a total of 37 cycles. Template DNA was initially denatured at 94°C for 1 min and then subjected to 16 cycles of 92°C for 30 s, 50–0.5°C per cycle for 30 s, and 72°C for 2 min, followed by 21 cycles of 92°C for 1 min, 40°C for 1 min, and 72°C for 2 min; the final step was 5 m at 72°C. Reaction products were electrophoresed on 1.5% agarose gels containing 3 µl of 10 mg ml^{-1} ethidium bromide. The gels were run for 5–6 h at 60–70 V and visualized using a UV transilluminator. Allele sizes were determined using RFLPSCAN ver. 2.1 gel analysis software (Scanalytics, Billerica, Mass.). The association of polymorphic RAPD markers with the *N. africana* chromosome was verified by genotyping 24 BC_1F_1 individuals derived from the cross NC152-*dhfr* -996/McNair 944// McNair 944. These plants were also phenotyped for resistance to Mtx. Cosegregation analysis was performed on these genotypic and phenotypic data.

Isolation and characterization of $2n=48/PVY^R$ plants

For BC_1F_1 families possessing putative introgression events, an additional 24 plants were tested for resistance to Mtx and PVY NN. The chromosome number and meiotic pairing were determined for each PVY^R plant by examining pollen mother cells at metaphase I according to Burns (1982). Determinations were made on at least eight countable cells per genotype. To estimate the relative amounts of *N. africana* chromatin present in $2n=48/PVY^R$ types, three to five $2n=48/PVY^R$ individuals per family were genotyped at those RAPD loci where polymorphism existed between NC152-*dhfr* -996

and the bulk of three $2n=48/\text{PVY}^S$ lines. Preference was given to the selection of $\text{Mtx}^S/2n= 48/\text{PVY}^R$ plants, although one $\text{Mtx}^R/2n=48/\text{PVY}^R$ plant was included per family. Each of the $2n= 48/\text{PVY}^R$ plants that was genotyped was also crossed as a male and as a female to PVY^S var. K326 to determine the pollen and ovular PVY^R transmission rates. One hundred plants from each cross were inoculated with PVY^{NN} at the two-leaf stage and evaluated using previously described methods.

Marker-assisted backcrossing

Progenies from crosses between a selected $\text{Mtx}^S/2n=48/\text{PVY}^R$ BC_1F_1 plant and K326 were used to initiate backcrossing of the PVY resistance factor to K326 and to determine the potential for using molecular markers for the selection of genotypes possessing reduced amounts of *N. africana* chromatin. The BC_1F_1 plant was crossed both as a male and a female to K326 and 40 progeny (20 from each cross) were initially genotyped. Only those RAPD markers that were present in the parental BC_1F_1 plant were screened on progenies.

Results

Segregation of Mtx and PVY resistance in BC_1F_1 families

One hundred and forty-four BC_1F_1 families were generated using the non-tissue culture scheme. The percentage Mtx^R plants in each family was used as a preliminary screen to identify putative interchromosomal recombinatory events. The mean percentage survival was 7.6% with a range of 0.0–49.6%. While most all of the families exhibited *dhfr* transmission rates in the range of what was expected based on previous experience (Fig. 1), one family had a percentage survival that was conspicuously higher. The 49.6% survival rate in this family suggested that *dhfr* might have been transferred to a *N. tabacum* chromosome. This family is hereafter referred to as BC_1F_1 Family#1.

One hundred and forty-one BC_1F_1families were generated from tissue culture of 121 different NC152-*dhfr*-996 leaf discs. Consequently, the group consisted of at least 121 independently derived BC_1F_1 families. Average percentage survival was 9.2%, with a range 0.0–49.6%. Six families exhibited notably higher percentages (40.5–49.6%) of Mtx^R individuals (Fig. 2). These six percentages were also interpreted as possibly being caused by transfers of the *N. africana* chromosome segment bearing *dhfr* to a chromosome of the *N. tabacum* genome. Each of these six families was derived from a different leaf disc and are hereafter referred to as BC_1F_1 Families#2–7. A Chi-square test indicated that the frequency of putative introgression events obtained using the tissue culture scheme was significantly higher than that observed for the non-tissue culture scheme ($\chi^2=25.9$, $P<$ 0.001).

To gain insight into whether the PVY resistance gene was also transferred to a *N. tabacum* chromosome in BC_1F_1 Families#1–7, 100 plants from each family were tested for both resistance to PVY NN and Mtx. The percentage transmission of PVY-resistance to the BC_1F_1individuals in these families ranged from 38% to 53% (Table 1), which is consistent with the resistance gene residing on a *N. tabacum* chromosome. The frequency of recombination between *dhfr* and the PVY resistance gene ranged from $r=0.12$ to $r=0.24$ (Table 1). These results were highly supportive of the hypothesis that a chromosome segment bearing both *dhfr* and the PVY resistance gene had been transferred to a *N. tabacum* chromosome in each of the seven families and that it was possible to obtain crossing over within the introgressed segments.

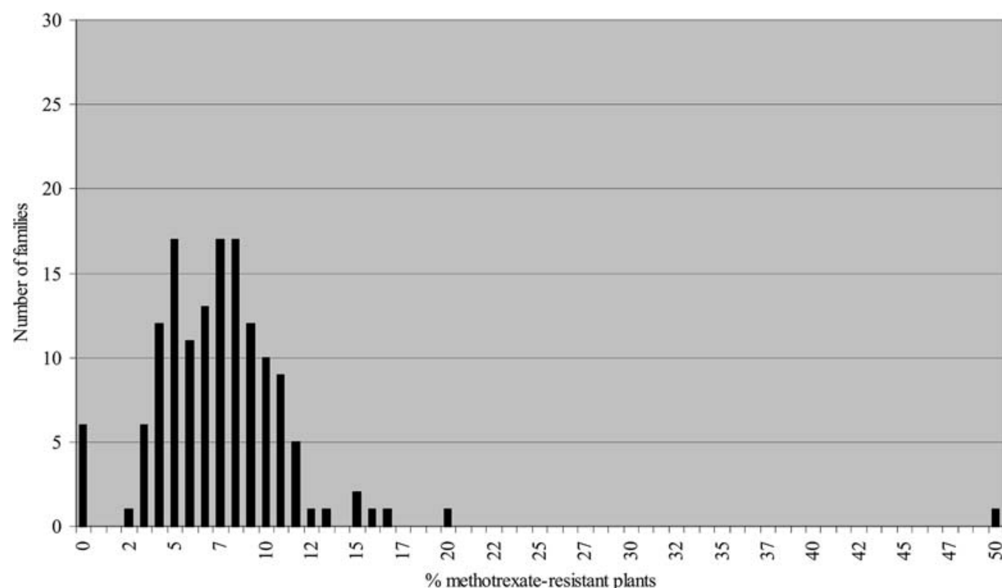

Fig. 1 Percentage methotrexate (Mtx)-resistant plants for the 144 BC_1F_1 families generated using a scheme that did not involve tissue culture

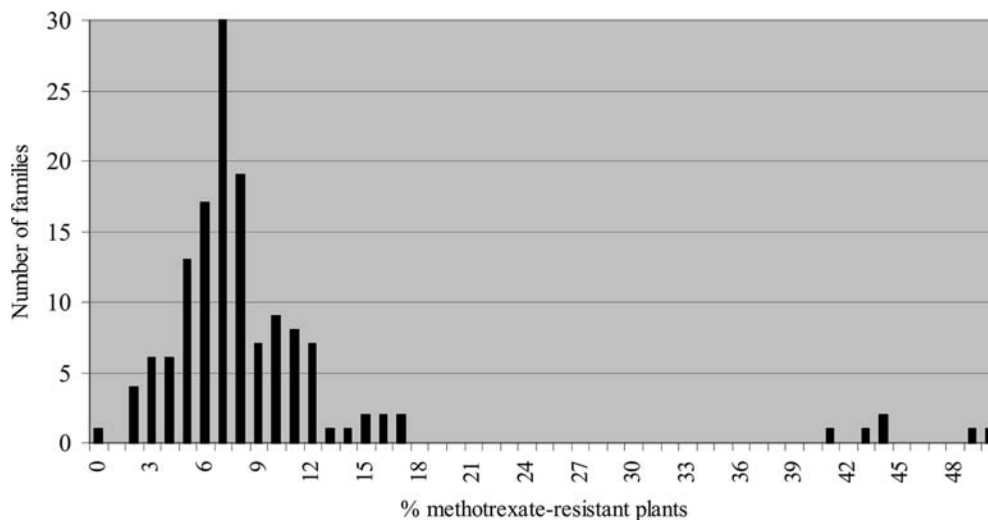

Fig. 2 Percentage Mtx-resistant plants for 141 BC_1F_1 families generated using a scheme that involved exposure of NC152-*dhfr*-996 explants to tissue culture

Identification of RAPD markers associated with the intact *N. africana* addition chromosome

Of 1,216 random decamer primers, 85 were initially identified that amplified a band that was present for NC152-*dhfr* -996 but absent for the $2n=48$ bulk (McNair 944, Petite Havana, K326). Based upon repeatability and ease of interpretation, 48 primers that amplified 51 markers were selected for further use (Table 2). Twenty-four BC_1F_1 individuals from the cross NC152-*dhfr* -996/McNair 944//McNair 944 were then tested for the presence of these 51 RAPD markers and for *dhfr* . Complete cosegregation was observed between all RAPD markers and *dhfr* . These data, in combination with the strong segregation distortion that was observed (2 Mtx^R : 22 Mtx^S), were consistent with all markers residing on the addition chromosome.

Isolation and characterization of $2n=48/PVY^R$ plants

$PVY^R/2n=48$ plants were isolated from BC_1F_1 Families#1–7. Almost all PVY^R plants possessed 48 chromosomes. While most $2n=48$ plants had cells exhibiting 24 bivalents (Fig. 3a), cells with 23 bivalents + 2 univalents were also observed (Fig. 3b). Three to five $PVY^R/2n=48$ individuals from each family were scored for the presence or absence of each of the 51 *N. africana* RAPD markers. Variability was observed among these plants with respect to the total number of *N. africana* RAPD markers that were present (range: 10–51, Fig. 4). Mtx^R plants possessed much greater numbers of *N. africana* markers (range: 45–51) than did Mtx^S plants (range: 10–25). Simple selection against *dhfr* apparently allowed for the elimination of a large fraction of alien germplasm. Based on these marker data, it was not possible to determine whether there were substantial differences among the seven introgression events with respect to the amount of *N. africana* chromatin that was initially transferred to the *N. tabacum* genome. Almost all of the genotyped plants had unique marker profiles, suggesting that a fair amount of crossing over was occurring within the introgressed segments. This statement might be biased, however, as preference was given to genotyping Mtx^S plants. The frequency of PVY^R/Mtx^S plants within the BC_1F_1 families was actually low (Table 1), and it is

Table 1 Segregation of resistance to methotrexate (Mtx) and PVY^{NN} in selected BC_1F_1 families

BC_1F_1 family	Number of plants[a]				Segregation ratio/χ^2 value[a,b]			
	Mtx^R/PVY^R	Mtx^R/PVY^S	Mtx^S/PVY^R	Mtx^S/PVY^S	$Mtx^R:Mtx^S$	$\chi2$	$PVY^R:PVY^S$	$\chi2$
# 1	38	12	12	38	50:50	0.00	50:50	0.00
# 2	32	6	6	56	38:62	5.76*	38:62	5.76*
# 3	43	10	10	37	53:47	0.36	53:47	0.36
# 4	39	6	13	42	45:55	1.00	52:48	0.16
# 5	35	11	8	46	46:54	0.64	43:57	1.96
# 6	43	5	10	42	48:52	0.16	53:47	0.36
# 7	33	14	10	43	47:53	0.36	43:57	1.96

* Indicates significant deviation from a 1:1 ratio at the 0.05 probability level.

[a] Mtx^R, Methotrexate-resistant; Mtx^S, methotrexate-susceptible; PVY^R, PVY-resistant; PVY^S, PVY-susceptible

[b] Chi-square tests were used to test for significant deviations from an expected 1:1 ratio.

Table 2 Selected primers amplifying RAPD markers specific to the *Nicotiana africana* addition chromosome

Primer	Primer Sequence 5′ to 3′	Size of RAPD fragment (bp)
UBC2	CCTGGGCTTG	704
UBC18	GGGCCGTTTA	826
UBC18	GGGCCGTTTA	720
UBC78	GAGCACTAGC	941
UBC98	ATCCTGCCAG	931
UBC104	GGGCAATGAT	463
UBC119	ATTGGGCGAT	927
UBC134	AACACACGAG	832
UBC135	AAGCTGCGAG	1,068
UBC138	GCTTCCCCTT	539
UBC149	AGCAGCGTGG	301
UBC154	TCCATGCCGT	798
UBC202	GAGCACTTAC	520
UBC245	CGCGTGCCAG	767
UBC262	CGCCCCCAGT	628
UBC313	ACGGCAGTGG	335
UBC335	TGGACCACCC	547
UBC349	GGAGCCCCCT	701
UBC415	GTTCCAGCAG	706
UBC420	GCAGGGTTCG	491
UBC517	GGTCGCAGCT	651
UBC518	TGCTGGTCCA	825
UBC523	ACAGGCAGAC	880
UBC550	GTCGCCTGAG	332
UBC646	GTCCACTTCC	1,262
UBC668	CCCGATTGAG	503
OPAG06	GGTGGCCAAG	913
OPAH17	CAGTGGGGAG	324
OPAL09	CAGCGAGTAG	648
OPI11	ACATGCCGTG	293
OPI16	TCTCCGCCCT	405
OPK11	AATGCCCCAG	556
OPN20	GGTGCTCCGT	1,209
OPO09	TCCCACGCAA	893
OPQ06	GAGCGCCTTG	944
OPQ11	TCTCCGCAAC	992
OPQ17	GAAGCCCTTG	951
OPQ20	TCGCCCAGTC	1,148
OPU06	ACCTTTGCGG	486
OPV08	GGACGGCGTT	790
OPW16	CAGCCTACCA	545
OPW17	GTCCTGGGTT	1,170
OPW17	GTCCTGGGTT	694
OPZ20	ACTTTGGCGG	342
OPAB02	GGAAACCCCT	911
OPAB17	TCGCATCCAG	700
OPAD19	CTTGGCACGA	444
OPAE07	GTGTCAGTGG	1,042
OPAE07	GTGTCAGTGG	444
OPAE11	AAGACCGGGA	439
OPAE17	GGCAGGTTCA	392

not known whether this is due to a close linkage between *dhfr* and the PVY^R gene or due to infrequent crossing over within the introgressed alien segments.

Each BC_1F_1 plant that was genotyped was also crossed both as a male and a female to K326 to determine the ovular and pollen transmission rates for PVY resistance. The average ovular transmission for the 26 BC_1F_1 plants was 39.9% (range: 25–52%) (Table 3). Pollen transmission was extremely low in most cases and averaged 7.0%. One plant, (BC_1F_1 Family#3, plant 65-8), transmitted PVY resistance through the pollen 48% of the time. This

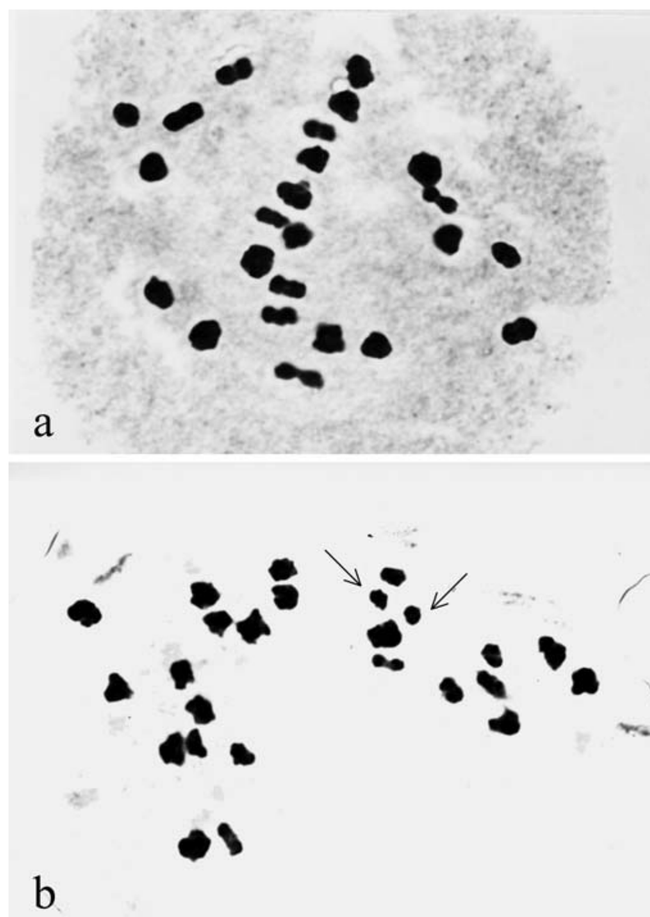

Fig. 3 Pollen mother cell meiotic chromosome associations at metaphase I. **a** Twenty-four bivalents exhibited by plant 7-20 (BC_1F_1 Family#7). **b** Twenty-three bivalents + 2 univalents (*arrows*) exhibited by plant 47-18 (BC_1F_1 Family#7)

data point was out of line with the other pollen transmission rates given the relatively high number of *N. africana* alleles (17) that were present for this plant. It is noteworthy that the two plants with the next two highest pollen transmission rates were those possessing the fewest *N. africana* RAPD markers. Plant 12-3 (BC_1F_1 Family#1) possessed only ten *N. africana* markers and had a pollen transmission rate of 39%.

Marker-assisted backcrossing

An experiment was undertaken to determine if the amount of *N. africana* chromatin present in BC_1F_1 plant 12-3 could be reduced further through molecular marker-assisted backcrossing. This plant was chosen because it possessed the least number of *N. africana* RAPD markers (ten) and exhibited a high transmission of PVY resistance through the pollen, indicating that it most likely carried the smallest introgressed *N. africana* segment. Twenty PVY^R plants from the cross plant 12-3/K326 and 20 PVY^R plants from the reciprocal cross were genotyped at those marker loci where a *N. africana*

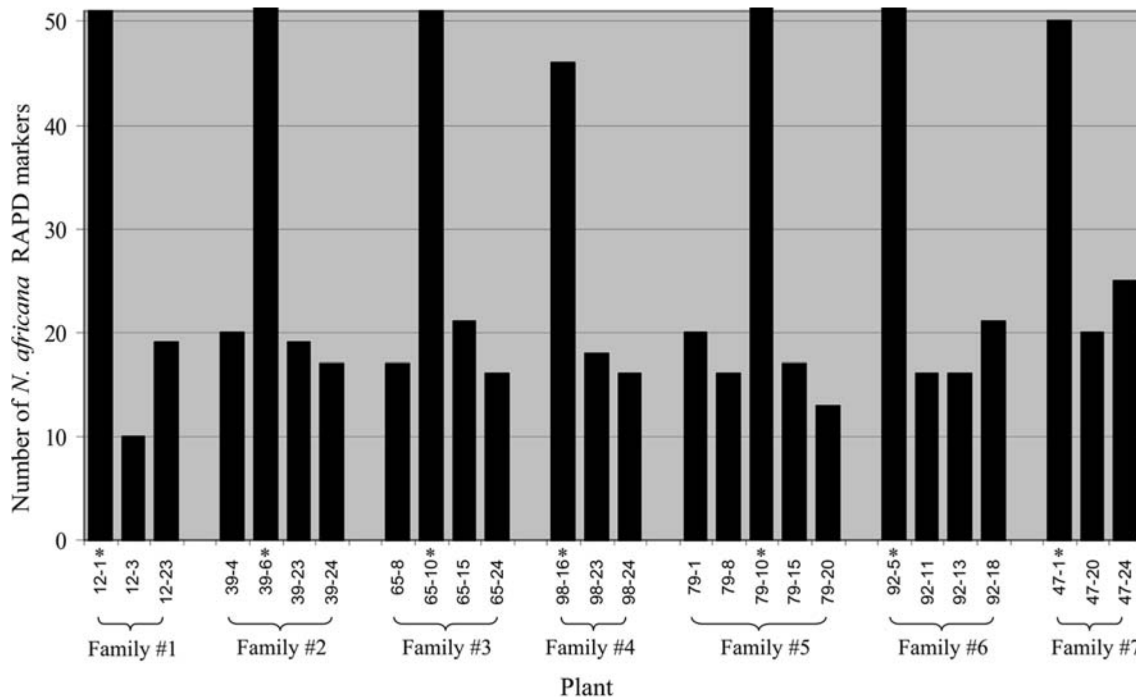

Fig. 4 Number of *Nicotiana africana* RAPD markers present in $2n=48$/PVYR individuals from seven BC_1F_1 families. Mtx-resistant plants are indicated with an *asterisk*

allele was present in plant 12-3 (in total ten loci). Of 40 plants, a single additional recombinant was identified that possessed only six of the 51 markers that were initially identified for the intact *N. africana* chromosome.

Discussion

The data presented in this paper demonstrate that a chromosome segment providing an increased level of resistance to a severe strain of PVY was transferred to tobacco from a *N. africana* addition chromosome in seven independent cases. The introgression lines do not exhibit the extreme type of resistance that is observed in *N. africana* per se or in F_1 hybrids between *N. africana* and *N. tabacum* . Instead, the introgression lines develop mosaic symptoms with no necrosis when inoculated with PVY NN. Multiple genes on several chromosomes may condition the full resistance that is observed in *N. africana* . Alternatively, the resistance factor may function differently within the genomic background of *N. tabacum*.

Six introgression events were obtained through a system involving tissue culture, and one event was obtained using conventional backcrossing without the use of in vitro culture. Banks et al. (1995) also observed an increased rate of transfer of a barley yellow dwarf virus resistance gene to wheat from a *Thinopyrum intermedium* (Host) Barkw. & D.R. Dewey addition chromosome using methods involving tissue culture as compared to a procedure using genetically induced homoeologous pairing. The data provided by these investigators do not reveal whether these gene introgressions occurred via translocation or simple homoeologous crossing over. The increased number of introgression events obtained from schemes that include the exposure of explants to tissue culture points to a possible role of tissue culture-induced chromosome breakage. The precise mechanism of such breakage is not known. Analyses of chromosome structural changes in tissue culture-derived plants have revealed that breakpoints are not random. In grass species, most tissue culture-induced breakages occur within centromeric chromatin or between heterochromatic knobs and the centromeres (McCoy et al. 1982; Lapitan et al. 1984; Johnson et al. 1987; Lee and Phillips 1987; Jorgensen and Anderson 1989; Hang and Bregitzer 1993). Such observations led to the hypothesis that late replication of heterochromatic regions in culture causes chromosome bridge formation and subsequent breakage (Lee and Phillips 1988; Peschke and Phillips 1992; Kaeppler et al. 2000). Late replication may be due to the disruption of normal cell-cycle controls that prevent cell division prior to the completion of DNA replication (Phillips et al. 1994). Changes in genome methylation status also occur during culture and may play a role in chromosome breakage (Phillips et al. 1994; Kaeppler et al. 1998).

The positions of the RAPD markers identified in this research relative to each other on the *N. africana* chromosome are not known. Few studies have examined the distribution of RAPD markers in *Nicotiana*, most likely because of a general lack of polymorphism in *N. tabacum* and a lack of interest in the non-cultivated species. A linkage map based on an interspecific *N. longflora* /*N. plumbaginifolia* population exhibited a fair

Table 3 Transmission of resistance to PVY^{NN} through the egg and pollen for selected $2n=48/PVY^R$ BC_1F_1 individuals

Individual	Ratio of PVY^R :PVY^S plants among progeny from the cross	
	BC_1F_1Plant/K326	K326/BC_1F_1Plant
BC_1F_1 Family#1		
Plant 12-1	52:48	8:92
Plant 12-3	45:55	39:61
Plant 12-23	38:62	1:98
BC_1F_1 Family#2		
Plant 39-4	46:54	4:96
Plant 39-6	46:54	0:100
Plant 39-23	25:75	2:98
Plant 39-24	39:61	4:96
BC_1F_1 Family#3		
Plant 65-8	47:53	48:52
Plant 65-10	41:59	3:97
Plant 65-15	32:68	2:98
Plant 65-24	49:51	0:100
BC_1F_1 Family#4		
Plant 98-16	41:59	0:100
Plant 98-23	39:61	4:96
Plant 98-24	40:60	5:95
BC_1F_1 Family#5		
Plant 79-1	41:59	4:96
Plant 79-8	46:54	0:100
Plant 79-10	45:55	4:96
Plant 79-15	39:61	0:100
Plant 79-20	26:74	28:72
BC_1F_1 Family#6		
Plant 92-5	48:52	2:98
Plant 92-11	39:61	0:100
Plant 92-13	28:72	0:100
Plant 92-18	38:62	0:100
BC_1F_1 Family#7		
Plant 47-1	45:55	2:98
Plant 47-20	48:52	0:100
Plant 47-24	48:52	1:99

distribution of RAPD markers across the genome (Lin et al. 2001). A *N. africana* mapping population would provide further insight on the distribution of our RAPD markers, but only a single *N. africana* accession is available. Although markers are most useful when their map positions are known, it was assumed that the RAPD markers in our case provided, at the least, a fair estimate of the *relative* amounts of alien chromatin present in the derived materials.

Marker data indicated that crossing over was occurring within each of the introgressed *N. africana* chromosome segments. This suggests that each segment was integrated into a position that permitted synapsis with a *N. tabacum* counterpart possessing at least some degree of structural and sequence similarity. Additional data (not shown) based on intercrosses among derived homozygous lines have since demonstrated that at least three of the seven introgression events involved the same *N. tabacum* recipient chromosome. Similar results were observed in work in wheat by Banks et al. (1995). Here, six of seven "tissue culture-induced" translocations involved the same recipient chromosome (Banks et al. 1995; Hohmann et al. 1996). These results lend support to the possibility that some form of homoeologous recombination was involved with these gene transfers. Tissue culture-induced translocations might be considered to be similar to irradiation-induced translocations in that they likely result from breaks and reunions between random or semi-random chromosomes. In vitro culture might also, however, influence introgression by providing circumstances that increase the opportunity for homoeologous crossing over (i.e. chromosome loss, alteration of factors controlling normal bivalent pairing, etc.).

The amount of apparent crossing over that was occurring within the introgressed segments was unexpected given the presumed taxonomic distance between *N. tabacum* and *N. africana*. Previous work found very little affinity between the chromosomes of *N. tabacum* and *N. africana* (Gerstel et al. 1979; Witherspoon 1987). *N. tabacum* is a $2n=48$ amphidiploid from section *Genuinae* that originated in South America (Goodspeed 1954), while *N. africana* ($2n=46$) is the only *Nicotiana* species to have been found on the continent of Africa. It has not been assigned to a section (Reed 1991), but cytological and biochemical observations suggest a relationship with the species of section *Suaveolentes* found in Australia and a few islands of the South Pacific (Merxmuller and Buttler 1975; Burk and Durbin 1978; Gerstel et al. 1979; Chen and Wildman 1981; Burns 1982). Evidence suggests a particularly close taxonomy with *N. fragrans* Hook. (Gerstel et al. 1979; Reed 1991).

The recombination that was occurring within the alien segments was used to our advantage. Molecular markers allowed the selection of PVY^R genotypes possessing relatively smaller amounts of alien chromatin. Without genotypic selection, it is unlikely that a PVY^R plant possessing this small number of *N. africana* markers would have been selected within the number of backcross generations that are usually used to transfer a disease resistance gene to an elite breeding line. Other investigations have demonstrated that recombination is often suppressed, but not necessarily prohibited, in chromosome segments introgressed from wild relatives (Paterson et al. 1990; Messeguer et al. 1991; Causse et al. 1994; Ganal and Tanksley 1996; Liharska et al. 1996). Additional effort may allow for further marker-based selection of PVY^R tobacco genotypes possessing minimal amounts of *N. africana* genetic material. Closely linked markers may also be valuable in work to pyramid this resistance mechanism with other resistance genes. With further development, derived materials may allow for an increased level and range of genetic resistance to PVY in tobacco.

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