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Construction of a designer chromosome in tobacco

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Abstract. The tobacco (*Nicotiana tabacum* L.) breeding line NC 152 is a doubled haploid that possesses an addition chromosome from *N. africana* [Merxm. and Buttler]. A gene on this chromosome confers potyvirus resistance ($Poty^R$). Our objective was to use the addition chromosome as a base on which to construct a designer chromosome containing a foreign gene linkage package. A mutant *dhfr* gene conferring resistance to methotrexate (Mtx) was inserted into NC 152-haploid (n = 25) leaf tissue via *Agrobacterium tumefaciens*-mediated transformation. After chromosome doubling, 135 NC 152*dhfr* transformants (2n = 50) were pollinated with the potyvirus-susceptible ($Poty^S$) cultivar 'McNair 944' (2n = 48). Linkage analysis was performed in the BC_1 generation. Two transformants, NC 152*dhfr*-996 and NC 152*dhfr*-1517, exhibited complete linkage between Mtx resistance (Mtx^R) and $Poty^R$. Segregants from these two transformants which were Mtx^R and $Poty^R$ possessed 49 chromosomes, while Mtx sensitive (Mtx^S) and $Poty^S$ progeny possessed 48 chromosomes. Eighty percent of the NC 152*dhfr* transformants transmitted the *dhfr* gene as one locus. Other foreign genes can be directed to the addition chromosome through transformation followed by selection for single loci with linkage to $Poty^R$ or Mtx^R. The integrity of both the foreign-gene linkage package and the rest of the genome will be maintained because recombination between the *N. africiana* and the *N. tabacum* chromosomes has not been observed.

Key words: *Agrobacterium tumefaciens* – *Nicotiana africana* – Methotrexate resistance – Potyvirus resistance – Linkage analysis

Introduction

Gene transfer mediated by *Agrobacterium tumefaciens* vectors has become routine in tobacco (De Block et al. 1987; Grierson et al. 1990; Hilder et al. 1990; Lindbo and Dougherty 1992), and the germ-plasm pool available for plant improvement now includes most living organisms. Comercial use of genetic transformation in agriculture depends on the incorporation of foreign genes into high-yielding germ-plasm. Plant breeders will probably want to combine several foreign genes into a single elite germ-plasm source. In order to accomplish this goal, we suggest designer chromosome construction.

Designer, or artificial, chromosomes have been produced in yeast. Yeast artificial chromosomes (YACs) contain all of the necessary functions for replication, including a centromere, autonomous replication sequence, and functional telomeres (Anand 1992), plus foreign genes of interest. In plants, the scaffold for designer chromosome construction can be found in a breeding line that possesses the full complement of chromosomes from its own species plus an addition chromosome from a related species. Addition chromosomes are often meiotically stable as homozygotes, and because recombination between addition chromosomes and the rest of the plant genome is rare (Gerstel 1945) the integrity of a foreign-gene linkage package will be preserved.

The placement of this linkage package on an addition chromosome will also minimize disturbance to the

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rest of the plant genome. The genetic structure of high-yielding germ plasm can be disturbed either through insertional mutagenesis or the disturbance of beneficial linkage blocks. Insertional mutagenesis, caused by the integration of foreign genes into plant coding sequences, can occur frequently. Koncz et al. (1989) estimated that at least 30% of all T-DNA insertions occur in transcribed regions of the *Arabidopsis* and *Nicotiana* genomes. Beneficial linkage blocks are formed through intermating and recombination followed by selection. They are the foundation for the superior performance of high-yielding germ plasm (Pederson 1974). If foreign genes are inserted into different parents, intermating will destroy linkage blocks. Breeders would like to be able to select for multiple foreign genes while minimizing the recombination that breaks beneficial linkage blocks.

The breeding line, NC 152 is a doubled-haploid chromosome-addition line (2n = 50) developed through interspecific hybridization of 'McNair 944' with *N. africana* (Wernsman 1992). The *N. africana* addition chromosome is stable and has been maintained through several generations of self pollination. Recombination between the addition chromosome and the *N. tabacum* chromosomes during meiosis has never been observed. The *N. africana* chromosome is sub-telocentric and visually distinct from the more metacentric tobacco chromosomes in haploid cell preparations (Fig. 1). When NC 152 is pollinated with normal (disomic) tobacco, 10% of the female gametes recieve the addition chromosome.

A gene on the *N. africana* chromosome confers resistance to at least two potyviruses, a necrotic strain of potato virus Y (PVY) (NC isolate 78, strain NN) (Lucas et al. 1980) and tobacco etch virus (TEV) (NC isolate 155A) (unpublished data, K. Campbell 1990). In F_2 progeny, the association between potyvirus resistance ($Poty^R$) and the presence of the addition chromosome is 100%.

Integration of T-DNA during *A. tumefaciens*-mediated transformation occurs throughout the genome. The location of foreign gene insertions has been determined through linkage analysis. Working with transgenic *Petunia hybrida*, Wallroth et al. (1986) searched for an association between kanamycin resistance and morphological markers located on each of the seven chromosomes to determine the location of the neomycin phosphotransferase (*npt*II) gene. In a similar linkage study, Bates and Zelcer (1992) identified a tobacco transformant with *npt*II inserted on the H chromosome, 5 map units away from the *n* gene for resistance to tobacco mosaic virus.

The objective of this research was to use *Agrobacterium tumefaciens*-mediated transformation and linkage analysis to inititate designer chromosome construction on the addition chromosome of NC 152.

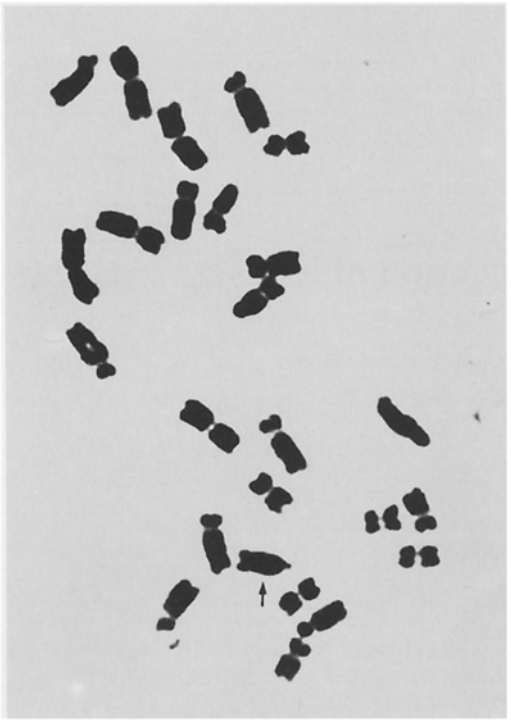

Fig. 1. Haploid karyotype of NC 152. The *arrow* is pointing to the addition chromosome from *Nicotiana africana*

Materials and methods

Transformation

Maternal haploids (n = 25) of NC 152 were generated through interspecific hybridization with *N. africana* (Burk et al. 1979). Leaf-disks excised from NC 152-haploids were transformed (An et al. 1986) with *A. tumefaciens* strain LBA 4404 possessing plasmid pWPF146 or pWPF147 (Fitzmaurice et al. 1992). Both plasmids contain the *dhfr* gene, which confers resistance to methotrexate (Mtx^R) (Simonsen and Levinson 1983). Transcription in these plants was driven by the cauliflower mosaic virus (CaMV) 35S promoter. The *dhfr* cassette was located within an engineered *Ds* element derived from the maize *Ac* element. This *Ds* element was inserted between the CaMV 35S promoter and the *β*-glucuronidase (GUS) coding sequence. Transposition of the *Ds* element in tobacco can be catalyzed by the introduction of the *Ac* transposase; excision of the *Ds* element results in the expression of GUS activity.

After 2 days of co-cultivation, the disks were transferred to MS-shoot induction medium comprised of MS inorganic salts and vitamins (Murashige and Skoog 1962) supplemented with 4.0 mg l^{-1} indole acetic acid (IAA), 2.5 mg l^{-1} kinetin, 30 g l^{-1} sucrose, and 10 g l^{-1} agar, plus 500 mg l^{-1} carbenicillin and 0.5 mg l^{-1} Mtx for the selection of transformed cells. Disks with green, Mtx^R callus were transferred to fresh medium every 14 days until shoots could be excised and placed onto MS rooting medium (MS inorganic salts plus 30 g l^{-1} sucrose and 10 g l^{-1} agar). We selected rooted shoots for a phenotype which, in our experience, gives rise to a high proportion of euploid plants of agronomic value.

The chromosome complement of several of the primary (R_0) NC 152*dhfr* transformants had doubled during shoot regeneration. These plants were fertile and produced seed in the greenhouse. Leaf midvein culture (Kasperbauer and Collins 1972) was used to double the chromosome complement and restore fertility to the remaining R_0 haploids. Selection for Mtx^R was imposed during the midvein procedure. Primary regenerants from the midvein procedure were also designated R_0.

Preliminary analysis

Progeny of 135 NC 152*dhfr* R_0 transformants were tested for Mtx and potyvirus resistance. Two years were required to complete resistance testing because of space limitations in growth chambers and greenhouses. The R_1, R_2, F_1, F_2, and BC_1 generations were developed for each R_0 transformant using $Mtx^R/Poty^R$ plants as females. These were either self-pollinated or pollinated with 'McNair 944', which is sensitive to both Mtx and potyvirus ($Mtx^S/Poty^S$). Three capsules were harvested from each cross.

Resistance to methotrexate

Resistance to Mtx was determined by germinating 60–120 seeds of each R_1, F_1, F_2, and BC_1 line in petri plates containing MS inorganics plus 1 mg l^{-1} Mtx and 10 g l^{-1} agar. Resistance in R_1 progeny was assessed 1 week after plating seeds onto Mtx. Sensitive seedlings germinated, failed to form roots, and subsequently died, while resistant seedlings formed healthy roots and first true leaves during this time period. The number of *dhfr* loci imparting resistance to Mtx was inferred from Mtx segregation ratios. The χ^2 test for a one-locus model with segregation ratios of 3:1 in the F_2 and 1:1 in the BC_1 was applied to each F_2 and BC_1 population derived from an R_0 transformant. A one-locus model was accepted if the χ^2 was less than 6.63 (prob $\chi^2 < 0.01$ with 1 df) for both the F_2 and BC_1 populations. A two-locus model was tested when the one-locus model was rejected.

Resistance to potyvirus

Three R_1 and 3 F_1 Mtx^R seedlings per R_0 transformant were transplanted into 10-cm-diameter pots for potyvirus resistance testing. Procedures for the inoculation and determination of resistance were as described by Witherspoon et al. (1991). As cool temperatures favor PVY symptom expression while TEV symptoms are more severe at hot temperatures (Lucas 1975), plants were inoculated with PVY (NC isolate 78, strain NN) from December through March and with TEV (NC isolate 155A) during the warmer months. We confirmed the resistance phenotype of the original NC 152-haploid plants used as explant sources by rooting axillary buds and inoculating them with PVY. Six Mtx^R BC_1 progeny per R_0 transformant were transplanted and inoculated with PVY. We used PVY to test segregating BC_1 progeny because phenotypic expression of resistance could be separated into distinct classes. Plants which lacked the addition chromosome exhibited veinal necrosis and death (PVY^S). Leaf mosaic and stunting was associated with the hemizygous state, while the homozygote exhibited only mild mosiac (PVY^R). Virus testing of BC_1 progeny was conducted in cool greenhouses or growth chambers (mean temperature of 17.6°–27.4 °C). 'McNair 944', NC 152, and the NC 152 × 'McNair 944' F_1 hybrid were included as checks in each test.

Analysis of NC 152dhfr-996 and *NC 152dhfr-1517 populations*

All 6 Mtx^R BC_1 progeny of R_0 transformants, NC 152*dhfr*-996 and NC 152*dhfr*-1517, were PVY^R. We performed three replications of Mtx and PVY resistance tests for each of these two lines. Tests were conducted as above except that all, rather than just 6, of the Mtx^R F_2 and BC_1 progeny were inoculated with PVY. In addition, we seeded 20 BC_1 progeny from each line directly into soil and inoculated them with PVY prior to testing for Mtx resistance. Because $Poty^S$ plants would be killed, apical stem cuttings from these 40 BC_1 progeny (20 from each line) were rooted prior to virus inoculation. Resistance to Mtx and chromosome counts were determined on the rooted cuttings. The similarity of Mtx segregation ratios between replications was determined by contructing χ^2 contingency tables (Briggs and Knowles 1967). Contingency tables were also constructed to determine similarity of Mtx segregation ratios between the F_2 and BC_1 populations, and between NC 152*dhfr*-996 and NC 152*dhfr*-1517 within populations. A χ^2 test was then employed to test segregation data against a model in which *dhfr* loci were transmitted to 10% of female gametes in these two lines.

Cytogenetic analysis

Chromosome counts were determined in five metaphase cells from corolla cell or root-meristem preparations (Burns 1982). Counts were made in the BC_1 generation of 4 $Mtx^R/Poty^R$ progeny and 4 $Mtx^S/Poty^S$ progeny for NC 152*dhfr*-996 and NC 152*dhfr*-1517. Chromosome numbers were also determined in 4 NC 152-haploids and in 4 R_1 progeny from each of 2 R_0 transformants whose progeny exhibited complete potyvirus susceptibility.

Results

We expected to obtain an insertion of the *dhfr* gene into the addition chromosome 4% of the time (1 per 25 chromosomes), assuming that insertion occurred as a single event, insertions were random into each chromosome, and chromosomes were of an equal size. Therefore, we calculated that 75 independent transformants were needed to have a 95% probability of obtaining 1 plant with *dhfr* located on the addition chromosome. To ensure that the R_0 plants were independent transformants, only 1 R_0 transformant per leaf disk was advanced to the R_1 generation.

Preliminary analysis

Chromosome doubling

Forty-two primary R_0 transformants were fertile. All progeny from 32 of these plants were Mtx^R, indicating that a haploid cell had been transformed and subsequently underwent chromosome doubling. The other 10 plants segregated for resistance to Mtx, indicating that chromosome doubling had occurred prior to transformation. We did not perform linkage analysis on these 10 plants. The chromosome complement of 93 additional haploid primary transformants was doubled through midvein culture.

Resistance to methotrexate

In our hands, Mtx resistance has proven to be a much more effective selectable marker than kanamycin resistance. Fewer that 1% of the total plants regenerated were Mtx^S. The *dhfr* gene appears to be dominant in its expression. We routinely obtained segregation ratios of 3 Mtx^R to 1 Mtx^S seedlings in the F_2 generation. Most of the R_0 transformants exhibited simple (one or two locus) inheritance of the *dhfr* gene (Table 1). Of the 16 R_0 transformants which exhibited complicated inheritance for *dhfr*, 10 were fit to a one-locus model ($\chi^2 < 3.84$) for Mtx segregation in either the F_2 or BC_2 generation, but not both generations. Additional replication may clarify the inheritance of *dhfr* in these transformants. Four other R_0 transformants may possess more than two *dhfr* loci. Two R_0 transformants, NC 152*dhfr*-996 and NC 152*dhfr*-1517, were selected for further characterization (described below). Segregation of progeny from these two transformants did not fit a one- or two-locus model (Table 1), but a 1:10 ratio of Mtx^R resistant to Mtx^S seedlings was observed in the BC_1 generation (Table 2). Four R_0 transformants were classified as Mtx^R in the R_1 generation, but root formation in the F_1 was inhibited (Table 1). For these plants, separation of Mtx^R and Mtx^S classes in the BC_1 generation was difficult. Although Mtx selection had been imposed during midvein culture of all of these plants, we noted in a separate study that sensitive plants could be regenerated off of midveins at a low frequency even in the presence of Mtx (unpublished data, K. Campbell).

Resistance to potyvirus

Expression of resistance to PVY and TEV was 100% correlated for all progeny of all R_0 transformants. We separated tested lines into resistance classes by comparison with the checks: 'McNair 944', NC 152, or the 'McNair 944' × Nc 152 F_1 hybrid. Although potyvirus resistance testing occurred during a 2-year period, viral symptoms were uniform throughout the course of this study. We observed severe stunting and interveinal necrosis on susceptible plants when inoculated with either virus; PVY-infected plants eventually died. With TEV, plants hemizygous for the addition chromosome could not be easily distinguished from homozygous plants; both were characterized by mosaic and necrotic flecking of the leaves. Resistance classes were distinct in PVY-infected plants. In the rare cases (three times) when 'McNair 944' did not exhibit chracteristic $Poty^S$ symptoms, the test was discarded and repeated. We obtained both $Poty^R$ and $Poty^S$ R_0 transformants from leaf disks excised from the same NC 152-haploid. Several R_0 transformants were $Poty^S$ (Table 1) as determined through progeny testing. Chromosome counts made in 2 of these transformants indicated that they possessed only 48 chromosomes. The addition chromosome was probably lost during shoot regeneration. Changes in the chromosome complement, including aneuploidy, are prevalent during in vitro culture of polyploid species such as tobacco (Karp 1986), and the screening for the euploid phenotype imposed upon R_0 plants actually selected against an addition chromosome. Alternatively, the addition chromosome could have been lost upon the selfing the R_0 plants, although this is unlikely since it is genetically stable as a doubled haploid.

Table 1. Segregation data from preliminary analysis of NC 152*dhfr* populations

Number of *dhfr* loci	Potyvirus resistance in R_1 generation	Number of transformants	Percentage of total
1	Res	72	57.6
1	Sus	18	14.4
2	Res	13	10.5
2	Sus	2	1.6
Unknown	Res	13	10.4
Unknown	Sus	3	2.4
Unknown	Weak res in F_1 and BC_1	4	3.2
Total		125	100.0

Res, Resistant; Sus, susceptibble

Table 2. Segregation data illustrating linkage between *dhfr* and potyvirus resistance in two NC 152*dhfr* populations

Generation	Response to methotrexate Observed		Expected		Response to potyvirus[a] Observed	
	Res	Sus	Res	χ^2	Res	Sus
	NC 152*dhfr*-996					
R_1	91	0	–	–[c]	5	0
F_1	82	0	–	–	5	0
F_2	74	332	40.6[b]	30.52**	–[d]	–
BC_1	46	457	50.3	0.42	37	0
	NC1 52*dhfr*-1517					
R_1	130	0	–	–	4	0
F_1	148	0	–	–	8	0
F_2	69	293	36.2	33.02**	–	–
BC_1	37	327	36.4	0.01	36	0

** Significantly different from expected ratio at the 0.05 probability level

[a] Only Mtx^R individuals were inoculated with potyvirus

[b] Expected segregation ratios were determined based upon 10% transmission through female gametes

[c] χ^2 was not calculated for non-segregating populations

[d] Resistance to potyvirus was not determined in F_2 populations

Analysis NC 152dhfr-996 and NC 152dhfr-1517 populations

Although few Mtx^R progeny were observed in the BC_1 generation of NC 152*dhfr*-966 and NC 152*dhfr*-1517, we had no trouble identifying Mtx resistance classes. Healthy roots and first true leaves were formed on Mtx^R seedlings, while Mtx^S segregants germinated and died within 1 week. The χ^2 contingency tables indicated that similar Mtx segregation ratios were obtained in each of three replications, (data not presented) so the number of plants were totalled across Mtx resistance classes for the F_2 and BC_1 generations within each transformant (Table 2). Similar segregation ratios were also observed for the two lines within generations. When untransformed NC 152 is outcrossed, the addition chromosome is transmitted to 10% of the female gametes but rarely transmitted through the male gamete. Therefore, we expected a 1:10 segregation ratio in both the F_2 and BC_1 generations. In this study, however, segregation ratios in the F_2 and BC_1 generations were significantly different from each other ($\chi^2 = 28.27$), and the F_2 data fit a model in which transmission occurred through both the female and male gametes.

Four phenotypic classes were expected in the BC_1 generation if *dhfr* and the gene conferring $Poty^R$ segregated independently. The two recombinant classes would be absent, under a one-locus model, if the genes were linked. All populations other than those derived from NC 152*dhfr*-996 and NC 152*dhfr*-1517 contained the $Mtx^R/Poty^S$ recombinant class. Only the parental classes were observed in NC152*dhfr*-996 and NC 152*dhfr*-1517 progeny. All Mtx^R plants from the BC_1 generation of these two lines were also $Poty^R$ (Table 2). For each line, 20 BC_1 progeny were inoculated with PVY prior to selection on Mtx. All 40 progeny were susceptible to PVY, indicating that the transmission rate of the addition chromosome is low in the absence of Mtx selection.

Chromosome counts were determined on segregating BC_1 progeny. For both lines, 4 $Mtx^R/Poty^R$ segregants each possessed 49 chromosomes. Four $Mtx^S/Poty^S$ segregants from NC 152*dhfr*-1517 each possessed 48 chromosomes. For NC 152 *dhfr*-996, 3 of the $Mtx^S/Poty^S$ segregants possessed 48 chromosomes, while the fourth was a monosomic with 47 chromosomes. Chromosome loss is not unusual in tobacco (Smith 1979).

Discussion

The use of addition chromosomes as scaffolds for designer chromosome construction requires the ability to generate many independent transformants. The number of transformants needed depends upon the total number and, probably, the relative size of chromosomes. Although the chromosome number of tobacco is high compared to other plant species, we analyzed 135 transgenic lines and found two plants with the desired insertion. Our chances of hitting the target chromosome were improved because NC 152 is a doubled haploid and recombination between the addition chromosome and the tobacco genome is rare. Bates and Zelcer (1992) performed linkage analysis on only 68 R_0 lines and identified two transformants possessing the *npt*II gene on chromosome H. Their discovery was fortuitous because their R_0 transformants were hemizygous for the *npt*II gene and subject to homologous recombination

Certain efficiencies were associated with our testing procedures. The linkage analyses were conducted on easily observed phenotypic markers. Antibiotic resistance was determined on 1-week-old seedlings, and the disease resistance marker located on the *N. africana* chromosome could be scored on young plants. We transformed haploid tissue and obtained doubled-haploid transformants so that all members within an R_1 or F_1 line were genetically identical. Therefore, the number of seedlings and young plants scored could be minimized. Still, the transformation and linkage analysis required constant use of a full greenhouse and growth chamber for 2 years. Realistically, time and labor contraints to the insertion of multiple foreign genes into NC 152 are great. The process could be simplified through construction of designer chromosomes in *N. glutinosa*, a true diploid (n = 12) species of *Nicotiana* with a short life-cycle. Selectable markers would permit the rapid movement of chromosomes into commercial cultivars through interspecific hybridization and backcrossing.

The practical application of addition chromosomes in cultivar development may be limited. The utility of several breeding lines containing translocations derived from interspecific hybrids has been reduced because of undesirable linkages that are difficult to break. Most addition lines are outside the limits of commercial acceptability for flue-cured tobacco cultivars (Wernsman and Rufty 1987). In replicated trials, leaf yields of NC 152 were 12% lower than those of 'McNair 944', but this reduction may be due to anther culture employed during the development of NC 152 rather than the presence of the addition chromosome. Tobacco doubled haploids generated through one cycle of anther culture exhibit a 12–15% decrease in yields compared with the cultivars from which they were derived (Arcia et al. 1978). More importantly, quality characteristics of NC 152 are within acceptable ranges for commercial cultivars and may be superior to 'McNair 944' (unpublished data, E. Wernsman). In addition, if several foreign genes are targeted to a single

addition chromosome, insertional mutagenesis may work in the breeder's favor by inactivating genes with deleterious effects.

The composition of the foreign-gene linkage package can be controlled. Should a gene located in the linkage package become undesirable, it can be deleted through a backcross to the isogenic untransformed NC 152, followed by the selection of desired recombinants. The *Ds* element provides a means of recycling antibiotic resistance markers; the transposition of *Ds* away from the addition chromosome will also remove *dhfr* construct located within the *Ds* borders. An R_1 plant from both NC 152*dhfr*-996 and NC 152*dhfr*-1517 has been crossed to a line encoding the *Ac* transposase in order to demonstrate transposition of the *Ds* elements and subsequent expression of the GUS excision marker.

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