# Resistance to Tobacco Mosaic Virus and *Meloidogyne arenaria* in Fusion Hybrids Between *Nicotiana tabacum* and an *N. repanda* × *N. sylvestris* Hybrid

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## ABSTRACT

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*Nicotiana repanda* has resistance to many important tobacco diseases, but no tobacco cultivars are currently available that carry resistance genes derived from this species. In order to transfer resistance genes into cultivated tobacco (*N. tabacum*), *N. repanda* was first hybridized with *N. sylvestris*, a species that is cross-compatible with both *N. tabacum* and *N. repanda*. This *N. repanda* × *N. sylvestris* (R × S) hybrid was then hybridized with *N. tabacum* cv. NC2326 by protoplast fusion. Parental lines were transformed for resistance to two antibiotics, hygromycin or kanamycin, with *Agrobacterium tumefaciens* transformation vectors. Fused protoplasts were selected by their ability to grow and regenerate on medium containing both antibiotics. No fusion calli were recovered from 15 different fusion experiments between NC2326 and the R × S hybrid, but calli were recovered from six of 63 fusion experiments when NC2326 was fused with a chromosome-doubled R × S hybrid. Calli appeared 4–6 mo after fusion, compared to the 8–12 wk required to obtain fusion calli between two sexually compatible *N. tabacum* cultivars, NC2326 and Burley 21. To date, rooted plants have only been obtained from one fusion experiment; these were designated as E fusion hybrids. A total of 69 of these fusion hybrids were established in the greenhouse 17–24 mo after fusion. All resembled NC2326 in morphology, but all had resistance to tobacco mosaic virus (TMV) derived from the R × S hybrid parent. Of 12 fusion hybrids analyzed for isozyme patterns of glutamate oxaloacetate transaminase, all demonstrated hybrid isozyme patterns. All 69 plants flowered and were male sterile, and all were successfully backcrossed to NC2326. To date, $BC_1F_1$ plants of 27 of the 69 E fusion hybrids have been analyzed for resistance to TMV and race 2 of *Meloidogyne arenaria*. Only 25 of 270 $BC_1F_1$ plants tested showed increased resistance to *M. arenaria*, four of which had resistance equivalent to that of the R × S hybrid. By contrast, 164 of 226 $BC_1F_1$ plants had resistance to TMV. $BC_1F_1$ plants also segregated for morphological traits. $BC_1F_1$ plants were again male sterile, but were successfully backcrossed to NC2326. The efficiency of the dual antibiotic selection system for selection of fusion hybrids in vitro is also reported.

*Additional keywords*: root knot, somatic hybridization, transformation.

Breeding for disease resistance in tobacco (*Nicotiana tabacum* L.) is limited by the germ plasm available and the method of incorporating resistance genes. *N. repanda* Willd. ex Lehm. (Fig. 1) has been reported to be resistant to more tobacco diseases and pests than any other wild *Nicotiana* species (4,10,22,30,31). These diseases include anthracnose, black root rot, black shank, brown spot, frogeye leaf spot, powdery mildew, wildfire, mosaic, and those caused by cyst nematode and three different species of root-knot nematodes. In addition, *N. repanda* is resistant to larvae of the tobacco hornworm and budworm (31).



Numerous attempts to hybridize *N. repanda* with commercial tobacco cultivars have been largely unsuccessful because of chromosome differences or the uncovering of lethal genes. Foster (10) initially crossed 4*n* *N. repanda* with 2*n* *N. tabacum* but failed to obtain any surviving progeny. Kincaid (16) used a mixed pollination technique and reported obtaining a single hybrid that was identified solely on morphological characteristics. Pittarelli and Stavely (24) also crossed 4*n* *N. repanda* with diploid *N. tabacum* and reported production of hybrids with resistance to wildfire and frogeye. However, no resistant lines were developed from these crosses.

A method employed to overcome incompatibility between *N. repanda* and *N. tabacum* is the use of *N. sylvestris* Speg. & Comes to "bridge" the cross, since *N. sylvestris* can be sexually hybridized with both species (3). Using this technique, Lea et al (19) and

Stavely et al (31) obtained hybrids with resistance to *Meloidogyne javanica* (Treub) Chitwood. However, no stable lines were obtained from these crosses. Burk (3) and Gwynn et al (12) obtained hybrids between an *N. repanda-sylvestris* sesquidiploid and two tobacco cultivars, NC2326 and Coker 139. Some progeny of backcross lines derived from these crosses showed resistance to tobacco mosaic virus (TMV) and cyst nematodes (12) but no resistance to *M. javanica* or *M. arenaria* (6,7). Advanced generations derived from these lines showed evidence of chromosome instability (12).

Cell culture techniques also have been tried for hybridizing *N. repanda* and *N. tabacum*. Reed and Collins (26) and DeVerna et al (8) cultured fertilized ovules in vitro but observed seedling lethal necrosis when the hybrids were transferred to soil. Iwai et al (14) obtained one sterile, morphologically intermediate plant by ovule culture that was TMV-resistant. Shintaku et al (28) obtained sterile hybrids by ovule culture of gamma-irradiated pollen or ovules. Recently, Zhou et al (34) reported successful maturation of hybrids by a combination of stylar fertilization, ovule culture, and growth of plants in the presence of indoleacetic acid (IAA). Somatic hybridization between the two species also has been attempted (2,9,15,18) and has generally been less successful than ovule culture. Kubo (18) reported regeneration of somatic hybrids between *N. repanda* and *N. tabacum*; however, the hybrids that were obtained lost *M. javanica* resistance in backcross generations. Bates (2) reported recovery of two TMV-resistant plants following fusion between protoplasts of *N. tabacum* and gamma-irradiated *N. repanda* protoplasts.

In spite of the extensive attempts to transfer genes between these two species, no tobacco cultivars are currently available that carry resistance derived from *N. repanda*. *M. arenaria* and *M. javanica* have become increasingly prevalent in tobacco fields in North Carolina, and the development of resistant cultivars is highly desirable. In addition, there is a need for an alternative source of TMV resistance in flue-cured tobacco, since the present source, derived from *N. glutinosa*, is linked with undesirable quality characteristics. The objective of this study was to transfer disease resistance genes from *N. repanda* to the flue-cured tobacco cultivar NC2326. Somatic hybridization was chosen because tissue culture has been shown to circumvent lethality of $F_1$ hybrids in interspecific crosses (20). In addition, in order to circumvent some incompatibility problems, an *N. repanda* × *N. sylvestris* (R × S) hybrid was used as the resistant parent, and fusions were performed with the R × S hybrid at two ploidy levels.

In this study, fusion hybrids were selected in vitro by a dual antibiotic selection system. Parent lines were transformed for resistance to kanamycin or hygromycin with *Agrobacterium* transformation vectors, and hybrids were selected for resistance to both antibiotics. The usefulness of dual antibiotic selection for selecting fusion hybrids in vitro is also reported.

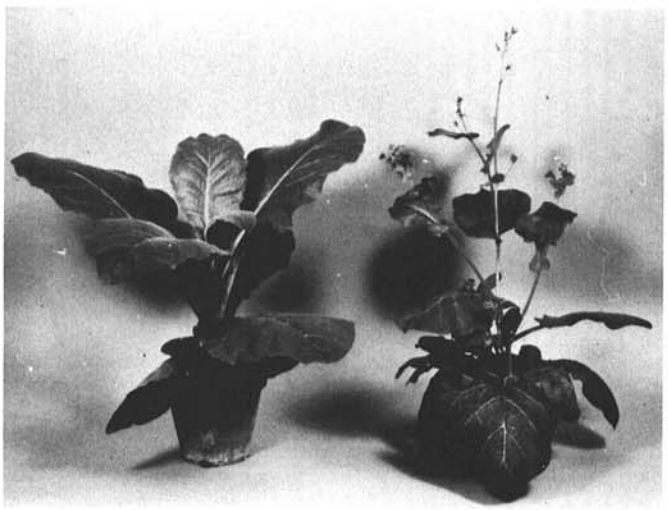

**Fig. 1.** Plants of *Nicotiana tabacum* cv. NC2326 (left) and *N. repanda* (right).

## MATERIALS AND METHODS

**Growth conditions.** Greenhouse plants were grown in a 2:1 (v/v) mixture of Metro-Mix, a commercial growing medium (W. R. Grace & Co., Cambridge, MA) and sterile soil containing Osmocote (an NPK 14:14:14 slow-release fertilizer; Grace Sierra Company, Milpitas, CA) and an NPK 3:9:9 fertilizer. Seedlings were transplanted 3 wk after seeding to 6.5-cm clay pots and later transferred to 15-cm clay pots. Greenhouse temperatures ranged from 24 to 34 C. Cell cultures and in vitro–grown plants were grown in a growth chamber at 25 C, with 14 h of light (75 $\mu E \cdot m^{-2} \cdot s^{-1}$) per day.

**Plant material.** *N. repanda* was pollinated by *N. sylvestris* under greenhouse conditions. Plants obtained from this cross (R × S hybrid) were transformed for hygromycin resistance (see below), and then the chromosomes were doubled to restore fertility and obtain seed. To double the chromosomes, disks cut from leaves of hybrid plants were cultured on solid .3/10 medium (Murashige and Skoog [MS] medium [23] with 0.3 mg of IAA, 10 mg of *N*-dimethylallyladenine, and 9 g agar per 1, adjusted to pH 6.0) to regenerate shoots. Developing shoots were treated with 0.3% colchicine for 2–3 h, followed by two sterile-water rinses. Colchicine-treated shoots were rooted on R5 medium (MS medium without hormones). Plants were then transferred to the greenhouse, grown to maturity, and selfed. In addition to the chromosome-doubled plants, *N. sylvestris* transformed with pCIB10 was crossed with untransformed *N. repanda*, and kanamycin-resistant progeny from this cross were used as fusion parents without doubling.

**Transformation.** *N. tabacum* cvs. NC2326 and Burley 21 and *N. sylvestris* were transformed for kanamycin and hygromycin resistance using a vector strain of *A. tumefaciens* (LBA4404) containing the plasmid pCIB10 (kanamycin resistance) or pCIB743 (hygromycin resistance) (27). Transformation was by the leaf disc method as described by Horsch et al (13). Transformed shoots were selected by culturing disks on MSBN medium (MS salts with 1 mg of pyridoxine, 1 mg of nicotinic acid, 1 mg of 6-benzyladenine, and 0.1 mg of naphthalene acetic acid per 1, adjusted to pH 5.7) containing either kanamycin (100 mg/L) or hygromycin (40 mg/L). Plantlets were rooted on R5 medium, and rooted plants were transferred to the greenhouse and selfed to obtain seeds. R × S hybrid plants transformed for hygromycin resistance with the vector strain pAT125 were provided by M. A. Conkling (North Carolina State University, Raleigh).

**Protoplast isolation.** Since antibiotic resistance segregated in progeny of transformed, selfed plants, surface-sterilized seeds from all parents were germinated in vitro on R5 medium containing either hygromycin at 40 mg/L or kanamycin at 100 mg/L. After 6 wk, antibiotic-resistant seedlings were transplanted to soil and grown in the greenhouse.

Protoplasts were isolated from fully expanded but young leaves of plants 8 wk after transplanting. Protoplasts were isolated essentially as previously described (15), except that protoplasts from R × S hybrid plants would not pass through miracloth, and thus that step was omitted.

**Protoplast fusion.** Fusions between three different sets of parents were made. *N. tabacum* cv. NC2326 transformed with pCIB743 (hygromycin-resistant) was fused with the R × S hybrid transformed with PCIB10 (kanamycin-resistant). NC2326 transformed with pCIB10 was fused with the chromosome-doubled R × S hybrid transformed with pAT125 (hygromycin-resistant). In addition, in order to test the efficiency of antibiotic resistance for selecting fusion products, Burley 21 transformed with pCIB10 was fused with NC2326 transformed with pCIB743. Protoplasts were fused using polyethylene glycol as previously described (15).

**Selection of fusion hybrids.** Protoplasts were suspended in liquid D3 medium (15) and incubated without selection at 25 C under a 14-h light and 10-h dark regime until cells regained cell walls and small callus colonies had formed (4–6 wk). The mannitol concentration of the medium was gradually decreased by weekly addition of D3 medium without mannitol. Small callus colonies

were then plated on 0.45-μm millipore filters (Millipore Corporation, Bedford, MA) on solid D3 medium without mannitol and containing both kanamycin (100 mg/L) and hygromycin (40 mg/L) (D3+K+H). Filters with calli were transferred to fresh solid D3+K+H plates every 2 wk. Surviving calli (beige or green) were individually transferred to solid .3/10 medium with kanamycin and hygromycin (.3/10+K+H). Regenerated shoots were detached from callus and allowed to root on R5 medium with IAA at 1 mg/L without kanamycin or hygromycin. Rooted plants were transferred to soil and grown in the greenhouse as described above under growth conditions. Plants were allowed to flower and were backcrossed by pollinating with NC2326.

**Isozyme analysis.** Approximately 10 mg of leaf tissue of putative fusion hybrids and 3-wk-old parents were ground in 10 μl of cold 0.1 M Tris-HCl buffer (pH 6.8). Following centrifugation, extracts were subjected to native polyacrylamide gel electrophoresis using the PhastSystem according to manufacturer's instructions (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The gels used were 12.5% homogenous gels with discontinuous buffer. The separation bed was initially cooled to 5 C prior to electrophoresis, and the sample was allowed to undergo separation for 35–40 min at 10 mA until the dye front had reached the anodic buffer strip end.

Sixteen isozyme stains were tested for differentiation of *N. tabacum* and the R × S hybrid. These were glutamate oxaloacetate transaminase (GOT), acid phosphatase, aconitase, alcohol dehydrogenase, alkaline phosphatase, amino peptidase, catalase, diaphorase, esterase, β-glucosidase, glutamate dehydrogenase, malic enzyme, phospho-glucomutase, phosphohexose isomerase, phosphogluconate dehydrogenase, and triose phosphate isomerase. Only GOT distinctly differentiated the two parents and thus was used throughout the study. For GOT isozyme staining, the gel was incubated overnight (25 C) on a shaker (26 rpm) in 50 mg of Fast Blue BB salt in 50 ml of GOT substrate solution (18.3 mg of α-ketoglutaric acid, 66.6 mg of L-aspartic acid, 250 mg of polyvinylpyrrolidone-40, 25 mg of $Na_2EDTA$, and 710 mg of $Na_2HPO_4$ per 50 ml, adjusted to pH 7.4) (5). After development, the gel was rinsed with deionized water prior to drying.

**TMV resistance trials.** TMV resistance was assessed on $R_0$ fusion hybrids and on progeny of the $R_0$ plants crossed to NC2326 ($BC_1F_1$ plants). TMV resistance of the $R_0$ fusion hybrids was assayed using a detached-leaf assay as previously described (15), except that boxes containing inoculated leaves were kept in a growth chamber at 25 C under 14-h light and 10-h dark conditions. With this assay, inoculated leaves of resistant plants develop local lesions after 3–4 days, whereas leaves from susceptible plants show no symptoms. A comparison of the detached-leaf assay with results from whole-plant inoculations on 60 plants of the Burley 21 × NC2326 fusion hybrids showed a 100% correlation between the two assays. Resistance of the $BC_1F_1$ plants was determined both by the detached-leaf assay and by whole-plant inoculations.

***M. arenaria* resistance trials.** Resistance to *M. arenaria* was assessed on $BC_1F_1$ progeny of the $R_0$ fusion hybrids crossed to NC2326. Ten plants derived from each E fusion parent were transplanted into a 1:1 sand–soil mix and arranged in one-plant replicates using a randomized block design. Plants were inoculated 2 wk after transplanting with freshly hatched stage 2 juveniles of a race 2 isolate increased on plants of Rutgers tomato. Juveniles were collected using the sodium hypochlorite and hatching chamber extraction method described by Barker (1). Inoculum was applied by injecting 1.5 ml of inoculum in each of two 3-cm deep holes located about 3 cm on either side of the plant. Inoculum also was placed in several randomly placed counting dishes, which were later counted to determine actual initial inoculum. Inoculum levels for experiments 1, 2, and 3 were 3,568 ± 203, 5,566 ± 299, and 5,151 ± 402 juveniles per pot, respectively. After 8 wk, roots were harvested, stained with phloxine B, and analyzed for both galls and egg masses. Gall ratings were determined on a 0–5 scale according to a modification of the rating scheme published by Zeck (33), with 0 = healthy and 5 = severely galled. Numbers of egg masses were also rated 0–5, with 0 indicating no egg masses and 1–5 indicating 1–2, 3–10, 11–30, 31–100, and >100 egg masses per root system, respectively (25).

## RESULTS

A total of 63 fusion experiments with NC2326 and the doubled R × S hybrid were performed, and in six of these, calli developed 4–6 mo following fusion on D3 selection medium containing both kanamycin and hygromycin. For each of the six experiments, there were between six and 10 tan or green callus clumps produced on selection medium over the 4- to 6-mo period, indicating six to 10 successful fusions out of the $5 \times 10^5$ protoplasts of each parent used in each fusion experiment. A total of 15 fusion experiments were performed with NC2326 and the nondoubled R × S hybrid. None of these gave rise to callus after plating on selective medium. There were 15 fusion experiments between NC2326 and Burley 21, 11 of which developed calli on selective medium 8–12 wk following fusion. In this case, numerous callus clumps developed following transfer to selection plates, so that filters were completely covered with fusion calli. In all experiments, unfused protoplasts of parental lines were also plated on media containing either kanamycin or hygromycin. In all cases, kanamycin-resistant parental protoplasts plated on hygromycin-containing medium failed to produce new cells and died after 2 wk, whereas those plated on kanamycin-containing medium showed prolific growth within 4 wk. Similarly, protoplasts of hygromycin-resistant parental lines failed to grow when plated on kanamycin-containing medium but grew well in the presence of hygromycin.

The six fusion experiments between NC2326 and the doubled R × S hybrid that yielded calli on selection plates were designated fusions A, B, C, D, E, and F. When calli were transferred to shoot regeneration medium containing kanamycin and hygromycin, callus from the E fusion was the first to produce shoots. Shoots produced initially were highly abnormal in appearance, with swollen, misshapen leaves, and did not survive transfer to rooting medium. After 10–12 mo, normal-appearing shoots developed that rooted within 4–10 wk following transfer to rooting medium. Rooted shoots derived from E fusion calli were transferred to the greenhouse 17–24 mo after fusion. To date, A and B fusion calli have also produced rooted shoots (at 38 and 33 mo, respectively, after fusion), but none have survived transfer to soil. By contrast, calli resulting from fusion of protoplasts

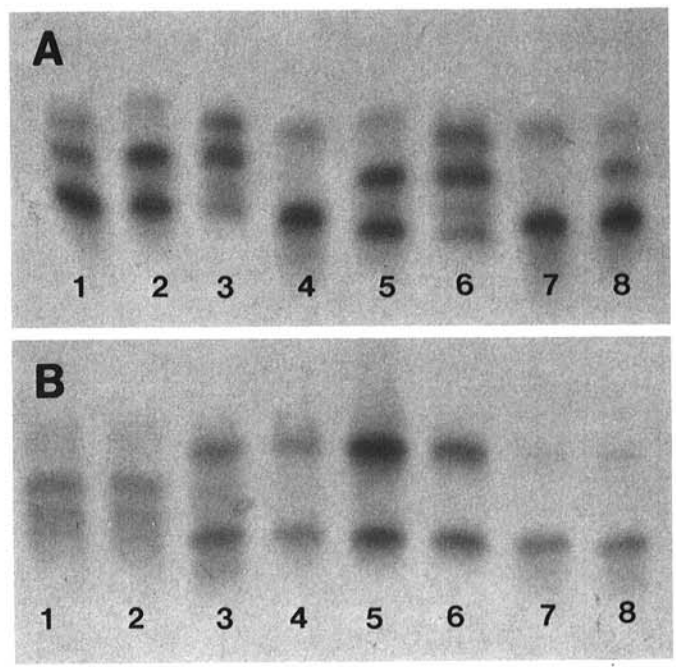


**Fig. 2.** Isozyme patterns of glutamate oxaloacetate transaminase of *Nicotiana tabacum* cv. NC2326, the *N. repanda* × *N. sylvestris* (R × S) hybrid and the E fusion hybrids produced from callus at **A,** 12 mo, and **B,** 18 mo, after fusion. **A,** R × S hybrid (lanes 2 and 5), E fusion hybrids (lanes 3 and 6), NC2326 (lanes 4 and 7), and mixture of extracts from R × S hybrid and NC2326 (lanes 1 and 8). **B,** R × S hybrid (lanes 1 and 2), E fusion hybrids (lanes 3–6), and NC2326 (lanes 7 and 8).

of compatible *N. tabacum* cultivars (NC2326 and Burley 21) developed shoots and roots within 8 and 4–6 wk, respectively, of transfer to appropriate media. Rooted plants of this fusion were transferred into the greenhouse 12–18 wk after fusion.

Nonrooted shoots and regenerated plants recovered from the E fusion callus 12–13 mo after fusion were tested for hybridity by GOT isozyme staining (Fig. 2A). Leaf extracts of fusion hybrids (lanes 3 and 6) showed banding patterns different from either parent (lanes 2, 4, 5, and 7) and from the banding patterns obtained when extracts from parental lines were mixed together (lanes 1 and 8). Both parental lines shared a faint upper band. In addition, the R × S hybrid (lanes 2 and 5) had a pronounced middle band and two lower bands that were not always resolved, and NC2326 (lanes 4 and 7) had a pronounced lower band that comigrated with the lowest R × S hybrid band. At 12–13 mo, the fusion hybrids (lanes 3 and 6) had all four bands of the two parents. However, the fusion hybrids could be clearly distinguished from the mixture of parent extracts (lanes 1 and 8) because the upper band was more pronounced in the fusion hybrids than in either parent or in the mixed extracts. Banding patterns of plants produced from callus 18 mo after fusion had changed (Fig. 2B, lanes 3–6). In these plants, the two middle bands derived from the R × S hybrid were lost, but hybridity was still indicated by the pronounced upper band. GOT isozyme banding patterns of NC2326, Burley 21, and the NC2326 × Burley 21 fusion hybrids were all identical (not shown).

A total of 81 E fusion plants were regenerated in culture, 69 of which survived transfer to soil. Morphologically, all 69 plants resembled the *N. tabacum* parent with large leaves, long internodes, and a terminal panicle with pink flowers. Twelve plants were tested by isozyme analysis, and all 12 showed hybrid banding patterns. All 69 surviving plants carried resistance to TMV derived from the *N. repanda* parent as demonstrated by the presence of local lesions in the detached-leaf assay. None of the plants were self-fertile, but all were successfully backcrossed by pollination with NC2326 pollen.

A total of 60 NC2326 × Burley 21 fusion hybrids were regenerated, and all survived transfer to soil. All 60 were shown to have resistance to TMV derived from the Burley 21 parent and to have green stems derived from the NC2326 parent instead of the white stems from the Burley 21 parent.

TABLE 1. Tobacco mosaic virus resistance of the $BC_1F_1$ progeny of E fusion hybrids × *Nicotiana tabacum* cv. NC2326

| Line | $BC_1F_1$ plants | | |
|---|---|---|---|
| | Number tested | Number resistant | % Resistant |
| 1 | 17 | 13 | 76 |
| 2 | 9 | 7 | 78 |
| 4 | 13 | 7 | 54 |
| 7 | 9 | 8 | 89 |
| 11 | 7 | 1 | 14 |
| 14 | 25 | 18 | 72 |
| 18 | 15 | 8 | 53 |
| 21 | 5 | 4 | 80 |
| 25 | 10 | 5 | 50 |
| 26 | 13 | 7 | 54 |
| 28 | 9 | 8 | 89 |
| 30 | 9 | 5 | 56 |
| 33 | 4 | 1 | 25 |
| 36 | 9 | 8 | 89 |
| 38 | 12 | 11 | 92 |
| 39 | 25 | 18 | 72 |
| 41 | 4 | 2 | 50 |
| 43 | 5 | 3 | 60 |
| 46 | 2 | 1 | 50 |
| 52 | 4 | 3 | 75 |
| 57 | 3 | 2 | 67 |
| 62 | 3 | 2 | 67 |
| 67 | 7 | 2 | 29 |
| 77 | 13 | 10 | 77 |
| 81 | 4 | 2 | 50 |

Unlike the $R_0$ plants, $BC_1F_1$ progeny of the E fusion hybrids with NC2326 were highly variable in morphological traits. Individual plants segregated for leaf shape and size, venation, internode length, and root morphology. Variation was noted more within lines than among lines derived from individual $R_0$ plants. All $BC_1F_1$ plants that flowered had a terminal panicle and pink flowers characteristic of NC2326.

$BC_1F_1$ lines were tested for TMV resistance, and all lines tested thus far are segregating for resistance (Table 1). Of the 226 individual $BC_1F_1$ plants tested, 164 were resistant to TMV.

Results of tests for resistance to race 2 of *M. arenaria* are shown in Table 2. NC2326 is highly susceptible to *M. arenaria* as indicated by the high gall and egg mass ratings in all three experiments. *N. repanda* and the R × S hybrid were both highly resistant and not significantly different from each other. $BC_1F_1$ plants varied in resistance. In order to obtain data on statistical differences, plants derived from each E fusion parent (10 plants per parent) were initially treated as a line and the data combined (Table 2). In all three trials, E fusion hybrid $BC_1F_1$ lines (E33, E18, E4, E41) were identified that were significantly more resistant than the NC2326 parent. Since the $BC_1F_1$ progeny are clearly segregating for morphological traits and TMV resistance, individual plants were also evaluated for resistance. The lowest 95% confidence limits for gall and egg mass ratings for NC2326 were 3.3 and 4.0, respectively, for all three trials. Thus a "resistant" plant was arbitrarily designated as any plant having both a gall rating of 2 or less and an egg mass rating of 3 or less. Of the 270 individual plants tested in the three trials, a total of 25 plants fell into the "resistant" category, four of which had gall and egg mass ratings of 0, equivalent to those of *N. repanda* or the R × S hybrid. The plants were saved and repotted, and axillary

TABLE 2. Mean gall and egg mass ratings of *Nicotiana tabacum* cv. NC2326, *N. repanda*, the *N. repanda* × *N. sylvestris* (R × S) hybrid, and the $BC_1F_1$ progeny of E fusion hybrids × NC2326

| Experiment | Line | Mean ratings[a] | |
|---|---|---|---|
| | | Galls | Egg masses |
| 1 | E30 | 4.3 | 5.0 |
| | E1 | 4.3 | 5.0 |
| | NC2326 | 4.1 | 5.0 |
| | E46 | 3.9 | 4.8 |
| | E21 | 3.8 | 4.8 |
| | E28 | 3.8 | 4.7 |
| | E14 | 3.7 | 4.9 |
| | E7 | 3.4 | 4.6 |
| | E33 | 2.9***[b] | 1.4*** |
| | E18 | 2.6*** | 2.4*** |
| | *N. repanda* | 0.5*** | 1.0*** |
| | R × S hybrid | 0.5*** | 0.7*** |
| 2 | NC2326 | 3.4 | 5.0 |
| | E52 | 3.2 | 4.7 |
| | E26 | 3.0 | 4.6 |
| | E43 | 2.9 | 4.6 |
| | E38 | 2.9 | 4.1 |
| | E25 | 2.9 | 4.7 |
| | E36 | 2.3 | 3.9 |
| | E2 | 2.3 | 4.3 |
| | E11 | 2.3 | 3.9 |
| | E4 | 2.2*** | 3.5*** |
| | R × S hybrid | 0*** | 0*** |
| 3 | E67 | 4.0 | 5.0 |
| | NC2326 | 3.9 | 4.6 |
| | E81 | 3.7 | 4.6 |
| | E77 | 3.6 | 4.3 |
| | E62 | 3.6 | 4.4 |
| | E66 | 3.6 | 3.7 |
| | E59 | 3.2 | 4.4 |
| | E57 | 3.1 | 3.7 |
| | E39 | 2.9 | 3.9 |
| | E41 | 2.7*** | 3.8*** |
| | R × S hybrid | 0*** | 0*** |

[a] Galls and egg masses were rated 0–5, with 5 being most severe.
[b] Significantly different from NC2326, using Dunnett's $t$ test, $P \leq 0.05$.

buds emerging from these plants were grown to flowering. Of these, 10 survived to produce new plants, and six have flowered. All six were male-sterile but were successfully backcrossed to NC2326.

## DISCUSSION

In this paper we report on the successful isolation of fusion hybrid plants derived from protoplast fusion between *N. tabacum* and an *N. repanda* × *N. sylvestris* hybrid. A total of 69 plants were successfully established in the greenhouse and grown to maturity. $R_0$ plants resembled *N. tabacum* in morphology, but presence of genes from the R × S hybrid was confirmed by isozyme analysis and TMV resistance. All plants were male-sterile but were successfully backcrossed to the *N. tabacum* parent. $BC_1F_1$ plants were identified that carried resistance to TMV and to race 2 of *M. arenaria*.

Parental lines and hybridization conditions chosen for this study were designed to optimize the likelihood of isolation of a large number of plants carrying genes for resistance derived from *N. repanda*. Numerous attempts have been previously made to hybridize *N. repanda* and *N. tabacum* either by conventional or cell culture approaches. Previous studies have mostly been unsuccessful or have resulted in the isolation of so few plants that desirable resistance traits could not be identified. In this study we attempted to combine factors shown in different studies to be advantageous. These included the use of an *N. repanda* × *N. sylvestris* hybrid as the resistant parent and the use of different ploidy levels, both shown to be important in studies on conventional hybridizations (3,19,24,31). We coupled these strategies with somatic hybridization. In vitro culturing of wide-cross hybrids has been shown to overcome lethality in highly incompatible crosses (20), presumably because of the large number of chromosome rearrangements that occur during growth in culture. Further, successful selection of desirable combinations of resistance and quality traits requires that we have large numbers of plants with which to work. To date, 164 of 226 $BC_1F_1$ plants tested were found to be resistant to TMV, but only four of 270 plants showed levels of root-knot resistance that were comparable to that of the R × S hybrid (with gall and egg mass ratings of 0). Somatic hybridization allows for the regeneration of large numbers of plants from hybrid cultures over time, whereas conventional crosses have resulted in the isolation of, at best, one or a few fertile plants (3,12,19,24,31).

Calli were obtained from only six of 63 fusion experiments between NC2326 and the doubled R × S hybrid on selective medium after 4–6 mo, whereas 11 of 15 fusion experiments between the sexually compatible NC2326 and Burley 21 resulted in callus development within 8–12 wk. No calli were obtained from fusion experiments between NC2326 and the nondoubled R × S hybrid. Time required for shoot and root production also differed significantly, with the NC2326 × R × S hybrid fusion calli requiring 16 mo or more for the production of rooted shoots as compared to 12–18 wk for rooted shoots from NC2326 × Burley 21. The low numbers of callus cultures and the long time period required for callus, shoot, and root formation from the NC2326 × R × S hybrid fusions indicate great genetic incompatibility in such a cross. Similar results have been reported with other wide-cross fusions, in which 12 and 16 mo, respectively, were required for plant regeneration from fusion calli between *Arabidopsis* and *Brassica*, and potato and tomato (11,21).

All 60 plants regenerated from the NC2326 × Burley 21 fusions were TMV-resistant and had green stems, indicating that antibiotic resistance markers are very efficient in selecting for fusion hybrids. The usefulness of dual antibiotic selection also has been reported by other investigators (17,29,32). The availability of an efficient selection scheme for selecting fusion hybrids in vitro has traditionally been a limiting factor in the establishment of successful fusion protocols. *Agrobacterium*-mediated transformation of antibiotic resistance genes coupled with dual antibiotic selection allowed us to overcome several problems encountered in a previous fusion study (15), in which we selected *N. repanda* × *N. tabacum* somatic hybrids on the basis of regeneration of light green (*Su/su*) plants following fusion of "sulfur" albino (*Su/Su*) mutants with normal green (*su/su*) protoplasts. Transformation is fast and easy compared to the lengthy efforts required to cross the *Su* marker gene into the desired parental lines. Secondly, antibiotic resistance allowed selection of fusion hybrids at the cellular level. This not only eliminates the labor-intensive regeneration of plants prior to fusion hybrid selection, but it is particularly advantageous for the selection of fusion hybrids that are slow to develop. The 4–6 mo required for the growth of R × S hybrid × NC2326 fusion callus points out the importance of selecting against parental protoplasts that would rapidly outgrow the fusion callus. Finally, there were no "escapes" with the antibiotic selection scheme. In our previous study (15), the albino *Su/Su* plants reverted at a high frequency in culture to the light green phenotype, resulting in the isolation and testing of a large number of plants that were not fusion hybrids.

The ultimate success of our efforts will depend in large part on the inheritance of the resistance traits. The resistance of the R × S hybrid to TMV and *M. arenaria* indicates that the genes coding for these traits are fully dominant. The high numbers (67% overall) of TMV-resistant plants in the $BC_1F_1$ population also suggest that TMV resistance is dominant and may be controlled by a single gene. The low numbers of resistant plants and the range of resistance responses of the $BC_1F_1$ plants to *M. arenaria* could be interpreted as indicative of the lack of a major gene for resistance. However, analysis of gene frequencies in our study is complicated by the significant loss and rearrangement of chromosomes that characterize in vitro cultures of wide-cross hybrids, and which presumably have occurred in our system. Genes that are linked to the insertion site of the marker genes are much more likely to be maintained than those which are not. It is possible that a major gene for *M. arenaria* resistance exists, but that it was not linked to the marker gene and was lost in most fusion hybrids because of chromosome loss. Resolution of this question will require further analysis of progeny of the few $BC_1F_1$ plants with *M. arenaria* resistance equivalent to that of the R × S hybrid parent.

We are continuing to test our remaining E fusion hybrid $BC_1F_1$ plants for resistance to TMV and *M. arenaria*. Since resistance to *M. arenaria* occurs at a lower frequency than TMV resistance, our strategy is to identify plants with nematode resistance first. These are then repotted, and axillary buds are allowed to develop and are tested for TMV resistance. Plants are allowed to flower and are both selfed (if possible) and backcrossed to NC2326. Seed recovered from these crosses will again be tested for TMV and *M. arenaria* resistance. We also intend to test plants for resistance to other pathogens, primarily *M. javanica*. We are continuing to maintain fusion callus and regenerated shoots from the A, B, C, D, and F fusion experiments and are working to establish rooted plants in the greenhouse. If plants are recovered from any of these callus lines, they will also be analyzed for hybridity and resistance. Our goal is to develop stable, fertile lines that have both disease resistance and acceptable morphological traits, which can be evaluated in a breeding program.

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