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ORIGINAL PAPER

The negative influence of *N*-mediated TMV resistance on yield in tobacco: linkage drag versus pleiotropy

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Abstract Resistance to tobacco mosaic virus (TMV) is controlled by the single dominant gene *N* in *Nicotiana glutinosa* L. This gene has been transferred to cultivated tobacco (*N. tabacum* L.) by interspecific hybridization and backcrossing, but has historically been associated with reduced yields and/or quality in flue-cured tobacco breeding materials. Past researchers have suggested the role of pleiotropy and/or linkage drag effects in this unfavorable relationship. Introduction of the cloned *N* gene into a TMV-susceptible tobacco genotype (cultivar 'K326') via plant transformation permitted investigation of the relative importance of these possibilities. On average, yield and cash return ($\$ ha^{-1}$) of 14 transgenic *NN* lines of K326 were significantly higher relative to an isoline of K326 carrying *N* introduced via interspecific hybridization and backcrossing. The negative effects of tissue culture-induced genetic variation confounded comparisons with the TMV-susceptible cultivar, K326, however. Backcrossing the original transgenic lines to non-tissue cultured K326 removed many of these unfavorable effects, and significantly improved their performance for yield and cash return. Comparisons of the 14 corresponding transgenic *NN* backcross-derived lines with K326 indicated that linkage drag is the main factor contributing to reduced yields in TMV-resistant flue-cured tobacco germplasm. On average, these transgenic lines outyielded the conventionally-developed TMV-resistant K326 isoline by 427 kg ha^{-1} ($P < 0.05$) and generated \$1,365 ha^{-1} more ($P < 0.05$). Although transgenic tobacco cultivars are currently not commercially acceptable, breeding strategies designed to reduce the amount of *N. glutinosa* chromatin linked to *N* may increase the likelihood of developing high-yielding TMV-resistant flue-cured tobacco cultivars.

Abbreviations
TMV Tobacco mosaic virus

Introduction

Disease resistance genes can be transferred from one species to another using so-called 'conventional' methods involving interspecific hybridization followed by backcrossing (Hadley and Openshaw 1980; Stalker 1980; Goodman et al. 1987). Parasexual techniques involving protoplast fusion have also been investigated for their potential in interspecific (Evans et al. 1981; Bates 1990; Sproule et al. 1991; Bui et al. 1992; Donaldson et al. 1995) or intergeneric gene transfer (de Vries et al. 1987; Dudits et al. 1987; Hinnisdaels et al. 1991; Kisaka and Kameya 1994; Ramulu et al. 1996). In the last 10–15 years, the utility of plant transformation for transferring disease resistance genes from one species or genus to another has also been demonstrated (Rommens et al. 1995; Whitham et al. 1996; Tai et al. 1999; Spassova et al. 2001; Lanfermeijer et al. 2004). All of these approaches can have negative influences on the commercial utility of derived materials, however.

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In materials possessing disease resistance genes transferred using interspecific hybridization or protoplast fusion, there are often unfavorable associations between the presence of a resistance gene and characteristics such as reduced yields. This might be due to pleiotropic effects of the resistance gene per se, or due to linkage drag effects caused by the presence of deleterious genes of alien origin linked to the gene of interest (Legg et al. 1981; Zeven et al. 1983; Friebe et al. 1996; Brown 2002). Suppressed recombination within introgressed chromatin (Paterson et al. 1990; Messeguer et al. 1991; Causse et al. 1994; Ganal and Tanksley 1996; Liharska et al. 1996) can make it difficult to alleviate linkage drag effects through backcrossing (Stam and Zeven 1981; Young and Tanksley 1989). This can also complicate efforts to distinguish between pleiotropic and linkage drag effects (Purrington 2000; Brown 2002).

Plant transformation methods can be used to bypass linkage drag effects by facilitating delivery of a desired gene into an elite genetic background without flanking sequences from the donor species. These techniques can also introduce undesirable side effects, however. Most transformation methods involve the use of various forms of tissue culture. Tissue culture is widely known to cause a host of mostly unfavorable genetic changes including chromosome structural alterations, deletions, single base pair mutations, and changes in DNA methylation (Larkin and Snowcroft 1981; Lee and Phillips 1988; Peschke and Phillips 1992; Kaeppler et al. 2000; Jain 2001). The transformation event per se can also be mutagenic. A foreign gene, whether introduced using *Agrobacterium tumefaciens* or biolistic methods, can be inserted into either coding or non-coding genomic DNA, possibly causing unintended side effects (Feldmann et al. 1989; Koncz et al. 1992). Indeed, transformation has been used to generate T-DNA mutant libraries for *Arabidopsis thaliana* (Koncz et al. 1992; Krysan et al. 1999; Alonso et al. 2003) and rice, *Oryza sativa* L. (Sallaud et al. 2003). Undesirable genetic variation has been reported among transgenic lines of several crop species (Dale and McPartlan 1992; Jongedijk et al. 1992; Brandle and Miki 1993; Malnoe et al. 1994; Whitty et al. 1994; Xu et al. 1999; Elmore et al. 2001; Barro et al. 2002). Bregitzer et al. (1998) observed, in barley, that the tissue culture-based transformation process appeared to induce greater variation than tissue culture in the absence of transformation.

No research has been published in which plant transformation has been used to evaluate the relative importance of linkage drag and pleiotropy on unfavorable effects associated with a plant disease resistance gene. Likewise, limited data exists on the relative importance of somaclonal variation and transgene insertional mutagenesis on undesirable effects commonly observed within populations of transgenic plants.

Tobacco, *Nicotiana tabacum* L., has been used as a model species in both interspecific hybridization and plant transformation research. The transfer of genetic resistance to tobacco mosaic virus (TMV) is a classic case of interspecific gene transfer in plant breeding. The single dominant gene *N* confers resistance to TMV via a hypersensitive response and was initially transferred to tobacco from *N. glutinosa* L. via interspecific hybridization followed by backcrossing (Holmes 1938; Gerstel 1945; Ternovsky 1941, 1945; Goodspeed 1942; Kostoff and Georgieva 1944; Kostoff 1948; Valleau 1952; Oka 1961). Reductions in yield and quality have been associated with the *N*-gene in flue-cured tobacco (Chaplin et al. 1966; Chaplin and Mann 1978). This has made it difficult to develop commercially viable TMV-resistant flue-cured tobacco cultivars. It is not known whether observed problems are due to linkage drag or pleiotropic effects of the *N*-gene per se. The *N*-gene was also the first plant virus resistance gene that was cloned (Whitham et al. 1994), and plant transformation has now been used to transfer the cloned version of the gene to both tobacco (Whitham et al. 1994) and tomato, *Lycopersicon esculentum* L. (Whitham et al. 1996).

Experiments involving transgenes present a method by which the relative importance of linkage drag and pleiotropy can be investigated. The first objective of this research was to use plant transformation to produce a set of transgenic lines of flue-cured cultivar 'K326' possessing single insertions of the cloned version of the *N*-gene under the control of its native regulatory factors. The second objective was to compare these materials for yield and quality with K326 isolines with and without the *N*-gene that was introduced using conventional interspecific hybridization and subsequent backcrossing. Introduced effects from somaclonal variation and transgene insertional mutagenesis can complicate such comparisons, however. A third objective was to evaluate the relative importance of somaclonal variation through comparison of the original transgenic lines with those derived by backcrossing these lines to non-transgenic K326 (with selection for TMV resistance) to reduce, or remove, genetic changes induced by tissue culture. This also allowed for a more accurate determination of the importance of linkage drag on yield and quality reductions in TMV-resistant flue-cured tobacco. The importance of transgene insertional mutagenesis, if any, was investigated through examination of variability among multiple independently transformed backcross-derived transgenic lines.

Materials and methods

Transformation

A doubled haploid approach was used to produce transgenic TMV-resistant lines of flue-cured tobacco cultivar K326 that

were immediately homozygous for all transgene insertions. Haploid leaf tissue was produced using the method of Burk et al. (1979) and transformed using *Agrobacterium tumefaciens* according to the procedure of An et al. (1986). The *Agrobacterium* strain LBA4404 possessed plasmid pTG34, bearing the selectable marker *npt*II and a 13.0 kb *Xho*I fragment from a genomic DNA clone containing *N* and its *cis* regulatory factors (Whitham et al. 1994). After 2 days of cultivation with the vector, inoculated leaf discs were transferred to shoot regeneration medium comprised of MS organic salts supplemented with 4.0 mg l^{-1} indole acetic acid (or 0.1 mg l^{-1} indole butyric acid plus 1.0 mg l^{-1} benzyl amino purine), 2.5 mg l^{-1} kinetin, 30 g l^{-1} sucrose, and 7 g l^{-1} agar. Also added were 250 mg l^{-1} cefotaxime, 200 mg l^{-1} vancomycin, and 100 mg l^{-1} kanamycin to eliminate contaminating bacteria and to select for transformed cells. Disks were transferred to fresh medium every 14–21 days. Regenerated shoots were transferred to rooting medium consisting of MS inorganic salts plus 30 g l^{-1} sucrose and 7 g l^{-1} agar. Rooted plants were transferred to soil-filled pots in a growth room. Regenerated plants expressing resistance to TMV were identified using a detached leaf test previously described by Rufty et al. (1987). Plants whose leaves exhibited the localized lesions of a hypersensitive response 4- to 5-days postinoculation were classified as TMV-resistant. Leaves that did not produce a hypersensitive response were classified as coming from TMV-susceptible plants. Resistant haploid plants were chromosome doubled using the midvein culture method of Kasperbauer and Collins (1972) to produce TMV-resistant K326 doubled haploid individuals referred to hereafter as R_0 transformants.

Molecular analysis

The number of transgene insertions present in TMV-resistant doubled haploid individuals was determined using Southern blots. Genomic DNA was extracted according to Rogers and Bendich (1985) and 5 mg DNA sample^{-1} was digested with *Xba*I according to manufacturer's recommendations. *Xba*I cuts at a single site within the T-DNA borders of pTG34 and within the *N* open reading frame. Digested DNA was electrophoresed and transferred to nylon membranes according to Sambrook et al. (1989). The probe was created by PCR amplification using primers (5′-ACCAGAATGATATGTTCCAC-3′) and (5′-GGACTCAACGTTAATTCTCTG-3′), and was double labeled with a α-^{32}P-dCTP and α-^{32}P-dATP using a random primed labeling kit according to manufacturer's instructions.

Development of advanced generations

Certified seed of flue-cured tobacco cultivar K326 (hereafter referred to as K326 Certified) was obtained from Gold Leaf Seed Company (Hartsville, South Carolina). TMV resistance was introduced into this cultivar using conventional methods by initially crossing K326 Certified with TMV-resistant flue-cured tobacco cultivar 'Coker 51'. This cultivar carries the *N*-gene on chromosome H of the tobacco genome (Lewis et al. 2005). After 12 backcrosses to K326 Certified with selection for TMV resistance, a TMV-resistant individual was self-pollinated. An individual $BC_{12}S_1$ plant found to be homozygous for the *N*-gene (identified through test crossing) was self-pollinated to produce $BC_{12}S_2$ seed of a stable, TMV-resistant isoline of K326. $BC_{12}S_3$ seed was used for research described in this paper (designated hereafter as K326 *NN* $BC_{12}S_3$).

All TMV-resistant R_0 transformants were self-pollinated to produce R_1 seed. Materials derived from 14 independent transformation events were selected for this research. These materials were selected based on the presence of single *N* transgene insertions (hereafter referred to as *N**) as revealed by Southern blot data. The selections were random outside of the requirement of single transgene insertions. To produce seed lots derived from each of these 14 transformation events, five R_1 plants per event were self-pollinated to produce R_2 seed. Equal amounts of R_2 seed per plant was bulked to produce 14 seed lots hereafter referred to as K326 *N***N** R_2 lines. This group of lines was expected to possess genetic changes induced by the tissue culture processes associated with transformation and chromosome doubling.

To produce corresponding seed lots in which the majority of tissue-culture induced genetic changes, if any, were removed, individual R_1 plants corresponding to the 14 selected transformation events were crossed to K326 Certified. After two subsequent backcrosses to K326 Certified with selection for TMV resistance, a single plant per transformation event was self-pollinated. One BC_2S_1 individual per transformation event found to be homozygous for *N** (identified through test crossing) was self-pollinated to produce a total of 14 BC_2S_2 seed lots hereafter referred to as K326 *N***N** BC_2S_2 lines. Under the assumption of random genomic distribution for any tissue culture-induced genetic changes, an average of 87.5% of these changes was presumed to have been removed in the BC_2S_2 lines.

Agronomic evaluation

Field evaluation of the 30 entries outlined above (K326 Certified, K326 *NN* $BC_{12}S_3$, 14 K326 *N***N** R_2 lines, and 14 corresponding K326 *N***N** BC_2S_2 lines) was conducted in a total of three environments in the absence of TMV infection: the Central Crops Research Station (Clayton, NC, USA) during 2005, and the Upper Coastal Plain Research Station (Rocky Mount, NC, USA) and Oxford

Tobacco Research Station (Oxford, NC, USA) during 2006. A randomized complete block design with four replications was used at each location. Each plot consisted of a single row with 22 competitive plants. Inter-row spacing was 1.14, 1.16, and 1.20 m at Clayton, Rocky Mount, and Oxford, respectively. Within-row spacing was 0.56 m at all locations. The end plants of each plot served as guard plants and were removed prior to harvest. Suggested management practices for flue-cured tobacco production were used at all three research stations.

Leaves were harvested in four separate harvests (primings) and flue-cured. Each priming was weighed to generate yield data, and Official USDA grades were assigned by a former USDA grader. A numerical reflection of cured leaf quality for each plot was generated using the 2006 North Carolina Flue-Cured Tobacco Grade Index (Smith and Fisher 2007). Value per hundred weight ($\$ \text{cwt}^{-1}$) was calculated based on average prices paid for standard grades during the 2006 growing season. Plot values for grade index and $\$ \text{cwt}^{-1}$ were calculated using a weighted average over all four primings. Fifty-gram cured leaf samples were prepared for each plot by compositing cured leaf from each priming on a weighted-mean basis. Oven-dried samples were ground to pass through a 1-mm sieve and analyzed for percent total alkaloids and percent reducing sugars (expressed as a percentage of dry weight) using the method of Davis (1976).

Data analysis

A combined analysis of variance appropriate for analyzing a randomized complete block design over environments (McIntosh 1983) was conducted using PROC MIXED of SAS statistical software version 9.1 (SAS Institute, Cary, NC, USA) (Littell et al. 1996). Each location × year combination was considered as a single environment. Entry means were produced using the LSMEANS statement.

The influence of the following possible sources of variation on measured traits were tested using *t* tests generated by ESTIMATE statements in PROC MIXED to test for significant differences between the following means (Littell et al. 1996):

Tissue culture-induced genetic variation:
K326 *N***N** BC_2S_2 lines (group mean) versus K326 *N***N** R_2 lines (group mean)

Linkage drag effects due to *N. glutinosa* genetic factors linked to *N*:
K326 *N***N** BC_2S_2 lines (group mean) versus K326 *NN* $BC_{12}S_3$ (entry mean)

Pleiotropic effects of *N*:
K326 Certified (entry mean) versus K326 *N***N** BC_2S_2 lines (group mean)

All possible pairwise comparisons were also made between entry means. This allowed for comparison of all transgenic entries to K326 Certified. Statistical differences between individual transgenic BC_2S_2 entries and K326 Certified might point to a negative influence of transgene insertional mutagenesis (or residual deleterious tissue culture-induced variation) in certain lines. This also allowed for tests to see if the performance of individual transgenic lines was improved significantly through backcrossing.

Results

Transformation

A total of 126 TMV-resistant doubled haploid R_0 plants were regenerated from 83 leaf discs of the K326 Certified genotype. Therefore, at least 83 independently-transformed lines were obtained. Southern blot data indicated that 31 TMV-resistant plants derived from 28 leaf discs possessed single insertions of the *N* transgene. Thirty-nine transformants derived from 33 leaf discs possessed two or more *N* insertions. Transgene copy number was not determined for the remaining transformants. R_2 and BC_2S_2 lines derived from 14 primary transformants possessing single *N* insertions were selected for agronomic evaluation. The 14 selected primary transformants were all derived from separate leaf discs in order to ensure the independence of the transformation events (i.e., they were not potential clones).

Agronomic evaluation

The fourteen K326 *N***N** R_2 lines, their corresponding *N***N** BC_2S_2 counterparts, and two checks (K326 Certified and K326 *NN* $BC_{12}S_3$) were evaluated in a total of three field environments for yield, quality, and cured leaf chemistry. Highly significant differences between environments were observed for all six of the measured traits (Table 1). Significant differences were observed between entries for yield, cash return, and percent total alkaloids (Table 1). Significant genotype × environment interaction was observed for percent reducing sugars.

The TMV-susceptible commercial cultivar K326 Certified was the highest yielding entry in this investigation and produced the second highest cash return (Table 2). The conventionally-developed TMV-resistant entry K326 *NN* $BC_{12}S_3$ was the lowest yielding entry in the experiment and also produced the lowest cash return (Table 2). This entry yielded less than K326 Certified (623 kg ha^{-1}, $P < 0.05$), produced a lower cash return ($1,858 ha^{-1}, $P < 0.05$), and exhibited lower percent reducing sugars (2.33%, $P < 0.05$) and significantly greater percent total alkaloids (0.38%, $P < 0.05$) (Tables 3, 4). No significant differences were observed between these two isolines for grade index or $\$ \text{cwt}^{-1}$.

Table 1 Analysis of variance for TMV-resistant entries and corresponding checks evaluated in a randomized complete block design in three North Carolina environments

Source	*df*	Mean squares					
		Yield (kg ha^{-1})	Grade index	Value (\$ cwt^{-1})	Cash return (\$ ha^{-1})	Total alkaloids (%)	Reducing sugars (%)
Environment	2	7,380,706**	6,609****	200,143****	91,692,588**	21.5****	1,003****
Rep (environment)	9	681,188	16	476	7,159,227	0.7	21
Entry	29	268,117***	7	338	2,740,006**	0.1*	5
Entry × environment	58	98,021	6	349	1,277,216	0.1	4*
Pooled error	261	105,065	6	270	1,155,376	0.1	3

*, **, ***, **** Indicates significance at $P = 0.05$, 0.01, 0.001, and 0.0001 levels, respectively

Table 2 Entry means for transgenic TMV-resistant K326 and checks evaluated in three North Carolina environments [ranked in order of increasing return (\$ ha^{-1})]

Entry	Measured trait					
	Yield (kg ha^{-1})	Grade index	Value (\$ cwt^{-1})	Cash return (\$ ha^{-1})	Total alkaloids (%)	Reducing sugars (%)
K326 *N N* $BC_{12}S_3$	2,503	85.1	298.57	7379.25	2.67	13.89
K326 *N***N** #3A, R_2	2,656	84.4	293.88	7693.82	2.47	15.81
K326 *N***N** #3B, R_2	2,726	84.7	293.68	7947.90	2.33	15.68
K326 *N***N** #30A, R_2	2,673	84.9	298.45	7955.44	2.40	16.10
K326 *N***N** #32A, BC_2S_2	2,734	84.5	293.64	8039.73	2.34	15.67
K326 *N***N** #11G, R_2	2,716	85.3	299.65	8132.63	2.46	17.03
K326 *N***N** #1A, BC_2S_2	2,749	85.0	297.10	8134.84	2.21	16.10
K326 *N***N** #11A, R_2	2,709	86.4	307.56	8246.48	2.45	15.50
K326 *N***N** #3B, BC_2S_2	2,759	85.8	302.12	8307.76	2.55	14.40
K326 *N***N** #32A, R_2	2,829	85.4	297.62	8363.50	2.33	15.68
K326 *N***N** #14B, R_2	2,817	85.5	300.61	8468.67	2.45	15.80
K326 *N***N** #21A, R_2	2,868	85.4	298.21	8498.83	2.30	15.28
K326 *N***N** #1A, R_2	2,893	85.1	295.43	8520.71	2.35	14.72
K326 *N***N** #30A, BC_2S_2	2,919	84.8	294.56	8546.48	2.40	15.34
K326 *N***N** #4B, R_2	2,828	85.7	302.78	8561.41	2.50	15.44
K326 *N***N** #14B, BC_2S_2	2,850	85.9	303.64	8600.39	2.42	16.35
K326 *N***N** #3A, BC_2S_2	2,918	85.5	299.34	8614.55	2.26	15.82
K326 *N***N** #13A, R_2	2,924	85.9	301.95	8717.65	2.34	15.91
K326 *N***N** #35A, BC_2S_2	2,978	84.2	294.18	8755.55	2.44	15.66
K326 *N***N** #11A, BC_2S_2	2,851	86.4	307.86	8768.60	2.50	15.19
K326 *N***N** #35B, R_2	3,010	84.8	297.07	8770.59	2.25	15.85
K326 *N***N** #32C, R_2	2,837	86.8	310.77	8831.55	2.29	15.14
K326 *N***N** #32C, BC_2S_2	2,965	85.9	303.97	8970.06	2.50	15.22
K326 *N***N** #4B, BC_2S_2	3,004	85.4	300.29	9009.13	2.25	15.49
K326 *N***N** #13A, BC_2S_2	3,045	85.7	300.99	9097.13	2.39	16.10
K326 *N***N** #21A, BC_2S_2	3,039	85.6	302.33	9109.27	2.40	15.76
K326 *N***N** #35A, R_2	2,891	87.7	316.15	9133.84	2.27	16.10
K326 *N***N** #35B, BC_2S_2	3,113	85.2	295.83	9167.48	2.46	14.75
K326 Certified	3,126	85.1	296.15	9237.35	2.28	16.23
K326 *N***N** #11G, BC_2S_2	3,104	85.0	300.51	9291.43	2.19	16.41
Overall mean	2,868	85.4	300.16	8562.40	2.38	15.61
LSD (0.05)	256	2.0	15.27	923.55	0.22	1.54
CV %	11	2.9	6.22	13.20	11.13	12.06

Table 3 Group means for transgenic TMV-resistant K326 and checks evaluated in three North Carolina environments

Entry/group	Measured trait					
	Yield (kg ha^{-1})	Grade index	Value (\$ cwt^{-1})	Cash return (\$ ha^{-1})	Total alkaloids (%)	Reducing sugars (%)
K326 *N N* $BC_{12}S_3$	2,503	85.1	298.57	7379.25	2.67	13.89
K326 *N*N** R_2 Group mean (14 entries)	2,813	85.6	300.99	8417.36	2.37	15.72
K326 *N*N** BC_2S_2 Group mean (14 entries)	2,930	85.3	299.74	8743.74	2.38	15.59
K326 Certified	3,126	85.1	296.15	9237.35	2.28	16.23
Overall mean	2,868	85.4	300.16	8562.40	2.38	15.61

Table 4 Comparisons of trait means for TMV-resistant materials and certified K326 evaluated in three North Carolina environments

Comparison	Difference[a]					
	Yield (kg ha^{-1})	Grade index	Value (\$ cwt^{-1})	Cash return (\$ ha^{-1})	Total alkaloids (%)	Reducing sugars (%)
K326 Certified versus K326 *NN* $BC_{12}S_3$	623 (< 0.0001)	−0.04 (0.9697)	−2.42 (0.7524)	1858.10 (0.0002)	−0.38 (0.0008)	2.33 (0.0036)
K326 *N*N** R_2 line average versus K326 *NN* $BC_{12}S_3$	309 (0.0021)	0.4 (0.5772)	2.42 (0.6666)	1038.11 (0.0033)	−0.30 (0.0004)	1.82 (0.0020)
K326 *N*N** BC_2S_2 Line average versus K326 *N*N** R_2 line average	118 (0.0014)	−0.2 (0.4352)	−1.25 (0.5442)	326.39 (0.0107)	0.01 (0.7279)	−0.13 (0.5400)
K326 *N*N** BC_2S_2 Line average versus K326 *NN* $BC_{12}S_3$	427 (< 0.0001)	0.2 (0.7856)	1.17 (0.8345)	1364.50 (0.0002)	−0.29 (0.0007)	1.70 (0.0038)
K326 Certified versus K326 *N*N** R_2 line average	313 (0.0019)	−0.5 (0.5424)	−4.84 (0.3990)	819.99 (0.0185)	−0.09 (0.2728)	0.51 (0.3691)
K326 Certified versus K326 *N*N** BC_2S_2 line average	196 (0.0468)	−0.2 (0.7461)	−3.59 (0.5228)	493.60 (0.1499)	−0.10 (0.2219)	0.64 (0.2630)

[a] Difference calculated by subtracting the latter group/entry mean indicated in the comparison from the former. Number in parenthesis indicates the probability of observing the given difference if the true means were equal. These probabilities was generated using *t* tests conducted by SAS

When compared to K326 *NN* $BC_{12}S_3$, the group of K326 *N*N** R_2 lines expressing transgenic TMV resistance exhibited higher yield (309 kg ha^{-1}, $P < 0.05$) (Tables 3, 4). This increased yielding ability translated into greater cash return (\$1,038 ha^{-1}, $P < 0.05$) (Tables 3, 4). Significant differences were also observed for this comparison for leaf chemistry, as the K326 *N*N** R_2 group exhibited significantly lower percent total alkaloids and significantly greater percent reducing sugars relative to K326 *NN* $BC_{12}S_3$.

The yield and cash return of the transgenic lines increased significantly after backcrossing to K326 Certified (increases of 118 kg ha^{-1} and \$326 ha^{-1} for K326 *N*N** BC_2S_2 lines relative to K326 *N*N** R_2 lines) (Tables 3, 4). The performance (yield and cash return) of 11 of the 14 lines was improved by backcrossing, although only two significantly. One line, K326 *N*N** #35A, exhibited a significant decline in quality as reflected by grade index and \$ cwt^{-1} after backcrossing.

The K326 *N*N** BC_2S_2 lines, as a group, outyielded K326 *NN* $BC_{12}S_3$ (427 kg ha^{-1}, $P < 0.05$) and provided for an increased cash return (\$1,365 ha^{-1}, $P < 0.05$) (Tables 3, 4). The K326 *N*N** BC_2S_2 group also exhibited significantly lower percent total alkaloids and significantly greater percent reducing sugars relative to K326 *NN* $BC_{12}S_3$.

Relative to the TMV-susceptible check, K326 Certified, the group of 14 K326 *N*N** BC_2S_2 lines exhibited slightly lower yields (196 kg ha^{-1}, $P = 0.047$) (Tables 3, 4). No other significant differences were observed between K326 Certified and this group of lines.

When comparisons were made between individual K326 *N*N** BC_2S_2 lines and K326 *NN* $BC_{12}S_3$, most were significantly higher yielding, produced a greater cash return, and exhibited lower percent total alkaloids and higher percent reducing sugars (Table 2).

In pairwise comparisons with K326 Certified, five K326 *N*N** BC_2S_2 lines retained significantly lower yielding ability (Table 2). No K326 *N*N** BC_2S_2 line was significantly

different from K326 Certified for $ cwt^{-1} or grade index. Relative to K326 Certified, three K326 *N**N** BC_2S_2 lines produced a significantly lower cash return, and one line had significantly altered percent reducing sugars and percent total alklaloids. Line K326 #11G, BC_2S_2 produced the greatest cash return, although this was not significantly greater than that for K326 Certified.

Discussion

There are numerous reports suggesting that disease resistance genes, or linked factors, can affect agronomic productivity (Brown 2002). In the research described here, the reduced yield and $ ha^{-1} that were observed for K326 *NN* $BC_{12}S_3$ relative to K326 Certified are consistent with previous reports describing this unfavorable association (Chaplin et al. 1966; Chaplin and Mann 1978). Previously reported reductions in quality as reflected by grade index or $ cwt^{-1} were not observed in the materials used in this study, however.

It was of interest to determine whether or not the negative association between TMV resistance and yield was due to pleiotropic effects of the *N*-gene per se, or due to linkage drag effects caused by the presence of unfavorable genes of *N. glutinosa* origin linked to *N*. On average, K326 genotypes possessing the *N*-gene introduced via transformation exhibited greater yielding ability and $ ha^{-1} relative to K326 carrying the *N*-gene introgressed using conventional methods. Data reported here illustrate the impact that tissue culture-associated factors can have on such comparisons, however. The group of fourteen K326 *N**N** R_2 lines produced significantly higher yield and cash return relative to K326 *NN* $BC_{12}S_3$, but exhibited significantly lower performance relative to TMV-susceptible K326 Certified. Taken alone, this data might suggest that both unfavorable linkage drag and pleiotropic effects were associated with *N*. After backcrossing the R_2 lines to K326 Certified, however, the performance of the transgenic TMV-resistant materials was significantly improved. This points to a probable negative impact of deleterious genetic changes induced by the tissue culture process. Opportunities for development of somaclonal variation may be increased in this research because of two periods of exposure of tobacco explants to tissue culture: (1) tissue culture of leaf discs during the transformation process, and (2) tissue culture of haploid leaf midveins during the chromosome doubling process. The relative potential of these two stages for accumulation of undesirable genetic changes is unknown.

The ultimate goal of plant transformation research is often the evaluation of generated materials for agronomic characteristics under field conditions. Tissue culture-induced variation, as demonstrated here, can confound evaluation of newly-derived transgenic materials. The work indicates a need for strategies and tools that permit transformation of crop plants without collateral damage to the plant genome. Methods of possible value are those that minimize, or do not require, time in tissue culture (Birch 1997), or that use seed (Feldmann and Marks 1987) or germline transformation (Bechtold and Pelletier 1998).

As a group, the 14 K326 *N**N** BC_2S_2 lines exhibited an average yield that was still slightly lower than the yield of K326 Certified (difference of 196 $\mathrm{kg\ ha}^{-1}$, $P = 0.047$). When individual comparisons were made, however, most K326 *N**N** BC_2S_2 lines were not significantly different from K326 Certified for any of the measured traits. Line K326 *N**N** #11G, BC_2S_2 produced the highest $ ha^{-1} of all the entries tested in this investigation. Several BC_2S_2 lines exhibited significantly reduced performance relative to K326 Certified, however. This could be explained by residual deleterious somaclonal variation in these lines. On average, 87.5% of such changes would have been removed after two backcrosses. A seemingly less likely possibility is that transgene insertional mutagenesis was contributing to observed deficiencies in some K326 *N**N** BC_2S_2 lines. It has been estimated that 10–40% of mutations in transformed *A. thaliana* are due to T-DNA insertions (Koncz et al. 1992). A large-scale experiment by Alonso et al. (2003) found nearly 59% of T-DNA insertions in *A. thaliana* to occur in gene sequences (i.e., promoter regions, coding exons, and 5′- and 3′-untranslated regions). Transgene integration into transcribed regions in *N. tabacum* might be assumed to be less likely because of greater amounts of non-coding DNA. The occurrence of measurable effects from insertion into active genes might also be less in *N. tabacum* because of genetic redundancy in the genome of this amphidiploid species. Further backcrossing and testing of certain transgenic lines could be used to resolve whether or not these observed deficiencies are due to transgene insertional mutagenesis or due to residual undesirable tissue-culture induced genetic changes.

The overall objective of this research was to investigate the nature of the negative association between TMV-resistance and yield in genetic materials carrying the *N*-gene on chromosome H of the tobacco genome. The general conclusion that can be reached is that linkage drag effects, rather than pleiotropy, contribute to the majority of the unfavorable effects on yield and cash return that have typically been observed in TMV-resistant flue-cured tobacco materials.

In principle, the deployment of transgenic TMV resistance offers the best potential for developing high-yielding, TMV-resistant flue-cured tobacco cultivars. Current strong international objection to transgenic tobacco cultivars would make commercialization difficult, however. The data described here point to the possible value of TMV-resistant

germplasm in which deleterious alien chromatin linked to the *N*-gene has been reduced, or to the possible value of germplasm carrying the resistance gene in an alternative genomic position. Lewis et al. (2005) presented results demonstrating that the *N*-gene has been introgressed into two chromosomes of the tobacco genome. Current US cultivars carry the *N*-gene on chromosome H. A group of accessions from the United States *Nicotiana* germplasm collection (Lewis and Nicholson 2007) carry the *N*-gene on an alternative chromosome. Experiments are currently underway to determine if yield and/or quality are more or less affected in flue-cured materials possessing the *N*-gene on the alternative chromosome. Lewis et al. (2005) also demonstrated that it is possible to obtain recombination within the introgressed *N. glutinosa* region when in either genomic position. Application of marker assisted backcrossing may permit for selection against undesirable *N. glutinosa* genes and may increase the probability of developing commercially-acceptable TMV-resistant flue-cured tobacco cultivars.

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