Theor Appl Genet (2007) 114:841–854
DOI 10.1007/s00122-006-0482-0

ORIGINAL PAPER

Analysis of an introgressed *Nicotiana tomentosa* genomic region affecting leaf number and correlated traits in *Nicotiana tabacum*

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Received: 30 July 2006 / Accepted: 6 December 2006 / Published online: 12 January 2007

Abstract Germplasm from closely related diploid relatives of tobacco (*Nicotiana tabacum* L.) could be of value for continued genetic modification of this species and for mapping quantitative trait loci (QTLs). We examined near isogenic tobacco lines and hybrids differing for an introgressed genomic region from *N. tomentosa* Ruiz and Pavon designated as *Many Leaves* that exhibits a large influence on leaf number and correlated traits. Within a 'Red Russian' genetic background, the region acted in an additive to partially dominant fashion to delay flowering time, and increase leaf number, plant height, and green leaf yield. Evidence of epistasis was observed as the region affected these traits to varying degrees in diverse near isogenic hybrids. Fifteen amplified fragment length polymorphism (AFLP) markers of *N. tomentosa* origin were mapped within a single linkage group of 34.5 cM using a population of 207 BC_1F_1 individuals segregating for *Many Leaves*. Composite interval mapping produced 2–LOD confidence intervals for likely QTL positions influencing leaf number (3.1 cM region), plant height (2.9 cM region), and days to flowering (3.3 cM region). These intervals were overlapping. Results demonstrate that genomic regions with large genetic effects can be transferred to tobacco from closely related diploid relatives, and that sufficient recombination within these regions may permit mapping of genes controlling quantitative traits. Materials and results described here may be useful in future research to gain insight on the genetic control of the transition from vegetative to reproductive development in *Nicotiana*.

Abbreviations

AFLP	Amplified fragment length polymorphism
IL	Introgression line
NIH	Near-isogenic hybrid
NIL	Near-isogenic line
QTL	Quantitative trait locus
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism

Introduction

Vegetative development in plants, including the number of leaves produced, is regulated by a complex network of signaling pathways that are controlled by genes and their interactions with environmental conditions such as photoperiod and temperature (Amasino 1996; Koornneef et al. 1998; Mouradov et al. 2002). The number of leaves produced is often closely tied to days to flowering. These traits are of interest for several reasons. From a biological standpoint, there is curiosity in understanding the factors that stimulate transition from vegetative development to reproductive growth. In a sense, these are 'adaptability' genes that contribute to reproductive success in specific environments. Mapping experiments have led to the cloning of genes involved in flowering response in a number of species such as *Arabidopsis thaliana*, barley, wheat, tomato, and rice (Yano et al. 2000; El-Din El-Assal et al. 2001; Yan et al. 2004; Abe et al. 2005; Turner et al. 2005; Wigge et al. 2005; Lifschitz et al.

Communicated by H. T. Nguyen.

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2006). Although rapid progress is being made in this intense area of research, much remains unresolved.

From a more applied point of view, there is interest in leaf number and flowering time because of their relationship with crop plant yields. Unlike most agronomic crop plants, tobacco is cultivated for its leaves rather that its reproductive parts. In cultivated tobacco, leaves continue to develop on a central stalk until development of the primary reproductive structures is initiated. Leaf number and days to flowering are consequently components of yield. Plant genetics that contribute to increased leaf numbers may aid in the development of higher-yielding cultivars (King 1986), and also play a role in alternative production regimes that de-emphasize harvest of leaves at lower stalk positions (Wernsman and Matzinger 1980).

Leaf number and flowering time are highly correlated traits in *N. tabacum* populations (Legg et al. 1965; Matzinger et al. 1966; Legg and Collins 1971; Pandeya et al. 1983). The species is generally considered to be day neutral or indeterminate, although spontaneous mutants of tobacco that flower only under short-day conditions do occur. Such mutants were used in initial experiments to investigate photoperiodism in plants (Allard 1919; Garner and Allard 1920). Outside of the two loci involved in this condition, leaf number and days to flowering have been found be polygenic traits, primarily under the control of genes with additive effects (Robinson et al. 1954, Matzinger and Mann 1962; Marani and Sachs 1966; Pandeya et al. 1983). The effect of different temperature and light regimes on these traits may be genotype-dependent (Kasperbauer 1966; Kasperbauer and Lowe 1966). There is interest in gaining increased insight on the genetic control of flowering time and its relationship with leaf number in *N. tabacum* for both theoretical and practical reasons.

A relative lack of polymorphism for RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), and RFLP (restricted fragment length polymorphism) markers within tobacco has limited their usefulness for mapping genes of *N. tabacum* origin. In crops such as tomato and rice, near isogenic lines (NILs) or introgression lines (ILs) possessing small segments of a wild relative's genome within the genome of the cultivated species have been useful for introducing DNA polymorphism, facilitating identification of favorable exotic alleles, and for dissecting corresponding quantitative trait loci (QTLs) (Eshed and Zamir 1996; Brouwer and St. Clair 2004; Fridman et al. 2004; Gur and Zamir 2004). NILs and ILs are identical for the entire genome except for a single introgressed region, and therefore all genetic variation between these lines is associated with the introduced segment. The use of such materials in molecular quantitative genetic studies increases the power of QTL identification and dissection by eliminating background effects of other segregating QTLs.

Chromosome segments from wild *Nicotiana* relatives have been introduced into cultivated tobacco mainly through efforts to introgress simply inherited disease resistance traits (Gerstel 1945; Clayton 1947; Valleau et al. 1960; Apple 1962; Clayton 1969; Lewis 2005). Relatively speaking, however, these gene donors are not closely related to the likely progenitor species of *N. tabacum* (*N. sylvestris* Spegazzini and Comes, and *N. tomentosiformis* Goodspeed), and recombination has often been suppressed in introgressed regions (Bai et al. 1995; Johnson et al. 2002; Lewis 2005; Lewis et al. 2005; Milla et al. 2005). While abundant marker polymorphism has been introduced in these transfers, the relative lack of recombination has limited the ability to map positions of valuable genes precisely within the introgressed regions.

Diploid species more closely related to *N. tabacum* (*N. sylvestris*, or species from section *Tomentosae*) might be valuable sources of alleles for improvement of quantitative traits in cultivated tobacco (Wernsman and Matzinger 1966). Introduction of germplasm from these materials should also introduce DNA polymorphism that might facilitate mapping of favorable genes derived from the exotic source. *N. tomentosa* Ruiz and Pavon ($2n = 24$), is a very large branching *Nicotiana* species that is native to Peru and Bolivia, and that has a high degree of taxonomic relationship to *N. tomentosiformis* ($2n = 24$), one of the probable progenitor species of *N. tabacum* (Goodspeed 1954; Chase et al. 2003; Knapp et al. 2004). Clausen and Cameron (1944) provided a brief description of a NIL of the tobacco line 'Red Russian' possessing an introgressed *N. tomentosa* chromosome segment on chromosome B. This region carries a genomic region designated as *Many Leaves* (*Ml*) that delays flowering by approximately 10 days and increases leaf number by nine relative to Red Russian in field environments in North Carolina (personal observations). These materials may be useful for investigating the potential of wild *Nicotiana* relatives as sources of genes for continued genetic modification of *N. tabacum*, for mapping loci involved in the control of quantitative characters, and also for gaining increased insight on the genetic control of the transition from vegetative to reproductive growth in *Nicotiana*.

The first objective of this research was to investigate the phenotypic effect of the introgressed *N. tomentosa* chromosome segment on leaf number and correlated traits when in heterozygous and homozygous condition in the Red Russian genetic background. The effect of

the introgressed QTL(s) was also examined in six diverse near isogenic hybrids (NIHs) that varied widely for plant type and leaf number. The third objective was to identify ALFP markers that were polymorphic between the two NILs and to use these to genotype a set of 207 field-grown BC_1F_1 individuals segregating for the introgessed chromatin. The goal was to identify markers linked to the introgressed flowering time QTL, to determine the degree of recombination between *N. tabacum* and *N. tomentosa* chromatin, and to more precisely map the genetic factor(s) influencing leaf number, plant height, and days to flower. If sufficient recombination occurs, diploid relatives closely related to *N. tabacum* might be valuable for molecular quantitative genetic studies in *Nicotiana*, and also as sources of alleles for continued genetic modification of the cultivated species.

Materials and methods

Plant materials

The genetic stock Red Russian *MlMl* was created by transferring an introgressed chromosome region originating from *N. tomentosa* to germplasm line Red Russian (hereafter referred to as Red Russian *mlml*) using an unreported number of backcrosses (Clausen and Cameron 1944). Seed of this NIL has been maintained by the North Carolina State University tobacco breeding and genetics program. Seed of the line Red Russian *mlml*, as well as two accessions of *N. tomentosa* (TW 140 and TW 141) were obtained from the United States *Nicotiana* Germplasm Collection (Oxford, NC). *Nicotiana tomentosa* accessions ITB 1015 and 914750067 were obtained from Altadis Institut du Tabac (Bergerac, France) and The Botanical and Experimental Garden of the Radboud University of Nijmegen (Nijmegen, The Netherlands), respectively.

In order to evaluate the genetic effect of the introgressed region on measured characteristics (see below) in a uniform genetic background, Red Russian *mlml* was crossed with Red Russian *MlMl* to generate F_1 seed designated as Red Russian *Mlml*. It was also of interest to determine the effect of the alien region in diverse F_1 hybrids with different genetic potentials for leaf number and flowering time. Therefore, both Red Russian *mlml* and Red Russian *MlMl* were crossed with Petite Havana, 'Connecticut Shade 8212', 'TN 86', 'NC 2326', 'Speight 168', and 'Xanthi' to produce six pairs of near-isogenic hybrids (NIHs). Petite Havana is a very early flowering genetic stock of tobacco with very low leaf number. Connecticut Shade 8212 is a cigar wrapper tobacco cultivar with high plant height and relatively high leaf number. TN 86 is a burley tobacco cultivar that flowers relatively late and has a high leaf number. NC 2326 and Speight 168 are flue-cured tobacco cultivars that produce, on average 16 and 22 leaves, respectively. Xanthi is an oriental tobacco cultivar that produces a relatively high number of smaller leaves on an average-size stalk.

Field evaluation of lines and hybrids

The effects of genetic factors within the *Many Leaves* region were tested in a total of three environments. The first two environments involved evaluation of a total of 21 entries in replicated field experiments at the Central Crops Research Station located near Clayton, NC, during 2003 and 2004. The third environment involved evaluation of 207 BC_1F_1 field-grown plants segregating for *Many Leaves* during 2005 (see below). The replicated field experiment during 2003 and 2004 included the following entries: Red Russian *mlml*, Red Russian *MlMl*, Red Russian *Mlml*, the six diverse tobacco lines described above, and the six NIH pairs. The experimental design each year was a randomized complete block design with three replications. Individual plots consisted of single, 5-plant rows. Rows were spaced 1.22 m apart with 0.53 m between plants within rows. Plants were decapitated (topped) one leaf below the lowest branch of the apical inflorescence at approximately the day of opening of the first flower. Suckers (lateral axillary branches) were controlled by hand approximately every other day after topping.

Data were collected for each plot for the following traits: average days to flower; leaf number; plant height (cm); leaf length (cm) for the 3rd, 8th (when present), and 13th (when present) leaves from the base of the plant; leaf width in cm for the same leaves; and green leaf yield (g $plot^{-1}$) at the point of plant maturity. Days to flower was based on the point when corollas were visible on 50% of the plants within the plot. Measurements for leaf number, plant height, leaf lengths, and leaf widths were made for each plant within the plot and averaged.

An analysis of variance was performed on collected data using PROC MIXED of SAS (SAS Institute Inc., Cary, NC). Entries were considered as fixed factors and years were considered as random. Entry means were calculated using the LSMEANS statement. PROC CORR was used to calculate Pearson correlation coefficients between measured traits. The genetic effects of the introgressed region on the measured traits within the Red Russian genetic background were estimated using entry means according to Edwards et al. (1987), where the additive value, '*a*,' was estimated as $a = (MlMl–mlml)/2$. The dominance value,

'*d*', was estimated as $d = Mlml–(MlMl + mlml)/2$. The dominant/additive ratio was calculated as d/a.

ESTIMATE statements were used in PROC MIXED of SAS to test for the following: (1) significant differences for measured characteristics between members of a NIH pair, (2) the significance of the effect of *Ml* over all seven genetic backgrounds (*Mlml* genotypes versus *mlml* genotypes), (3) significant differences between the effects of the introgressed QTL in different genetic backgrounds. Tests for significant heterosis for measured traits were also made for F_1 hybrid combinations using ESTIMATE statements in PROC MIXED of SAS according to Stalder and Saxton (2004).

Marker-trait associations

A total of 160 AFLP primer combinations (LI-COR, Lincoln, NE) were screened for their ability to reveal polymorphisms between DNA from Red Russian *mlml* and Red Russian *MlMl*. DNA was isolated according to Johnson et al. (1995), except that a BIO 101 FastPrep machine (BIO 101 Inc., Vista, CA) was used for tissue grinding. AFLP reactions were performed according Myburg and Remington (2000), and gels were run on a LI-COR model 4200 automated sequencer. Gels were scored initially by the software package AFLP-Quantar 1.0 (Keygene Products B.V., Wageningen, The Netherlands), and scoring was subsequently verified visually. Markers present for Red Russian *MlMl* but absent for Red Russian *mlml* were tentatively assigned to the introgressed chromosome region from *N. tomentosa*. Four accessions of *N. tomentosa* (specified above) were subsequently genotyped with these markers to provide insight on their origin. AFLP marker names were designated according to the primers used to amplify the DNA, followed by the band size in base pairs. E primers refer to those corresponding to *EcoR*1 restriction sites, while M primers refer to those corresponding to *Mse*1 restriction sites.

Polymorphic AFLP markers were subsequently mapped on BC_1F_1 individuals from the cross Red Russian *MlMl*/Red Russian *mlml*//Red Russian *mlml* that were grown in the field at the Central Crops Research Station during 2005. Plants were grown in five rows of 45 plants each (225 individuals total), with row and plant spacing as described above, and border rows surrounding the entire experiment. Days to flowering was recorded for each plant, and plants were subsequently topped as described above. At plant maturity, data were also collected for leaf number, leaf length (cm) and width (cm) of the 5th and 10th leaves from the base of the plant, plant height (cm), and green leaf yield (g). PROC CORR of SAS was used to determine phenotypic correlations between the measured traits. DNA was successfully isolated from 207 of the 225 plants. These plants were genotyped with those AFLP markers found to be present for Red Russian *MlMl*, but absent for Red Russian *mlml*. Chi-square tests (Steel et al. 1997) were applied to each marker to test for segregation distortion, and estimation of recombination fractions and linkage map construction were performed using MAPMAKER/EXP 3.0 (Lander et al. 1987). Linkage order was established by first selecting a subset of eight markers on the basis of LOD scores and pairwise linkages and then identifying the best order using the Compare command. Remaining markers were added using the Try command. The final marker order was checked by the Ripple command with the default log-likelihood threshold value of 2.0. Map distances (centimorgan, cM) were estimated from recombination fractions and the Kosambi mapping function (Kosambi 1944).

Polymorphic AFLP markers were subsequently tested for statistical associations with traits measured in the field-grown BC_1F_1 population. The GLM procedure of SAS was used to conduct single degree of freedom *F*-tests for each marker (Liu 1998) to determine if mean trait ratings differed significantly for the two AFLP genotype classes. Significance at the $P = 0.05$ level was considered evidence of linkage between the marker and a genomic region involved in the control of the trait. R^2 values were also calculated for each marker locus to determine that marker's contribution to the total variation for the measured traits. Composite interval mapping was also performed using Windows QTL Cartographer (Wang et al. 2001–2004). Log-likelihood (LOD) plots for statistically significant associations between markers and the measured traits were generated by calculating LOD scores at 0.5-cM intervals along the linkage group. LOD threshold significance levels ($\alpha = 0.01$ level) for each of the measured traits were determined using 1,000 permutations of the procedure of Churchill and Doerge (1994). 1-LOD and 2-LOD empiric confidence intervals for likely QTL positions were also determined using QTL Cartographer under the criteria of a 5 cM minimum distance between QTLs, and a minimum of 1 LOD from the top to the valley of a QTL peak.

Results

Field evaluation of lines and hybrids

Twenty-one entries were evaluated in a replicated field experiment during 2003 and 2004. Significant differences between years were detected for all measured traits except the length of the 13th leaf (Table 1). Very

Table 1 Analysis of variance for field evaluation of lines and F_1 hybrids with and without the introgressed *N. tomentosa. Many Leaves* genomic region

Source	*df*[a]	Mean squares									
		Days to flower	Leaf number	Plant height	Plot yield	Length of third leaf	Width of third leaf	Length of eighth leaf	Width of eighth leaf	Length of 13th leaf	Width of 13th leaf
Year	1 (1, 1)	427.2**	191.7***	4716.6*	73782726.7***	49448.5*	35040.0**	80761.4*	80359.3**	14516.1	57942.5***
Rep (year)	4 (4, 4)	14.0	2.1	417.4	790062.7	6179.1	980.9	8978.3	2887.6	2592.7	727.2
Genotype	20 (18, 14)	929.3****	336.9****	15317.7****	27317536.2****	46496.2**	31212.4**	52286.8****	18835.5****	41318.7***	19752.3***
Genotype × year	20 (18, 14)	116.3****	14.1****	1277.4****	2739013.7****	11986.8****	8064.4****	4907.3****	2721.5****	5328.0**	2402.8****
Pooled error	80 (72, 56)	6.7	2.0	278.7	315703.9	1624.9	540.5	966.4	411.4	2036.2	605.4

*, **, ***, **** Indicate significant differences at the 0.05, 0.01, 0.001, and <0.0001 levels, respectively

[a] Numbers in parenthesis represent degrees of freedom for analysis of variance concerning measurements on the eighth and 13th leaves, respectively

significant differences were also observed among entries for all measured traits. Significant genotype × year interactions were also observed for all measured characteristics. The interactions were mainly of the non-crossover type, and data were pooled over environments for presentation of entry means (Table 2).

Compared to Red Russian *mlml*, Red Russian *MlMl* flowered approximately 10 days later, produced almost nine more leaves, was approximately 63 cm taller, and produced 2,326 g more green leaf yield per plot (Table 2). The length and width of the third leaf of the *MlMl* genotype were greater relative to the same measurements for the *mlml* genotype. For the eighth leaf, the *MlMl* genotype had a greater leaf length and a smaller leaf width relative to the *mlml* genotype. Using the ESTIMATE statement of PROC MIXED in SAS to compare these two genotypes, only the differences for leaf number, plant height, and yield were found to be significant at the 0.05 level, however. Within the Red Russian genetic background, the introgressed region acted to influence all measured traits except the width of the third leaf and the length and width of the eighth leaf in an additive to partially dominant fashion (Table 3). Overdominance was present for these three traits.

By crossing the Red Russian NILs to each of six diverse lines, six pairs of NIH's were developed that allowed for comparison of the effect of the introgressed genomic region in backgrounds with different genetic potentials for flowering time and leaf number. In comparisons between members of a NIH pair, significant differences were observed for five of the six pairs for leaf number, four of six pairs for plant height, and for one pair for eigth leaf width (Table 4). There was a tendency for the introgressed region to more greatly increase leaf number and delay flowering in hybrids that had an increased genetic potential for later flowering and higher leaf number (hybrids involving TN86 and Connecticut Shade 8212). The effect of *Ml* on leaf number in hybrids involving Petite Havana was found to be significantly different than the effect observed for hybrids involving Connecticut Shade 8212 and TN86 (significantly different at the $P = 0.034$ and 0.005 levels, respectively). The *N. tomentosa* genomic region delayed flowering time and increased leaf number in hybrids involving Petite Havana (an early flowering, low leaf number genotype), but not significantly. *Mlml* genotypes had higher yields in all six NIH comparisons, but differences were not statistically significant in any case.

When compared over all F_1 hybrids, *Mlml* genotypes exhibited significantly delayed days to flower, and significantly greater leaf number, plant height, and plot

Table 2 Entry means for lines and F_1 hybrids with and without the introgressed *Many Leaves* genome region evaluated in two North Carolina environments

Genotype	Days to flower	Leaf no.	Plant height (cm)	Plot yield (g)	Length of third leaf (cm)	Width of third leaf (cm)	Length of eighth leaf (cm)	Width of eighth leaf (cm)	Length of 13th leaf (cm)	Width of 13th leaf (cm)
Red Russian *mlml*	45.3	11.6	73.2	3198	364.3	266.0	503.5	361.2	–	–
Red Russian *MlMl*	55.3	20.5	136.5	5524	474.7	327.8	555.9	354.3	561.0	368.5
Red Russian *mlml* × Red Russian *MlMl*, F_1	52.7	16.7	121.2	5278	473.3	337.5	564.2	373.9	552.2	391.8
Petite Havana	24.0	5.7	44.8	459	246.3	103.5	–	–	–	–
Petite Havana × Red Russian *mlml*, F_1	34.5	8.2	45.9	1895	359.0	207.7	–	–	–	–
Petite Havana × Red Russian *MlMl*, F_1	38.5	10.9	63.4	2344	372.0	212.3	486.8	287.7	–	–
Connecticut Shade 8212	70.3	29.6	221.4	5841	451.4	304.5	578.7	371.0	552.7	333.5
Connecticut Shade 8212 × Red Russian *mlml*, F_1	53.2	16.2	138.3	5564	467.0	335.7	634.5	414.7	612.8	404.2
Connecticut Shade 8212 × Red Russian *MlM*, F_1	64.5	25.9	210.9	6498	487.8	362.4	618.4	392.2	595.3	374.6
TN 86	80.2	34.2	176.8	7146	591.6	354.9	658.5	347.0	584.5	283.7
TN 86 × Red Russian *mlml*, F_1	53.5	16.6	117.8	7568	507.0	335.7	684.6	439.5	658.5	425.2
TN 86 × Red Russian *MlMl*, F_1	66.2	28.8	195.9	7788	545.0	358.2	677.7	415.3	651.2	394.5
NC 2326	59.0	17.4	101.9	6881	531.5	313.2	695.1	329.5	686.5	290.5
NC 2326 × Red Russian *mlml*, F_1	50.0	13.6	103.7	6551	488.0	339.0	669.9	425.5	–	–
NC 2326 × Red Russian *MlMl*, F_1	59.0	22.7	161.6	6727	524.7	370.8	621.9	360.3	646.7	377.5
Speight 168	63.5	23.9	110.4	7830	538.8	302.5	700.8	328.5	708.5	302.7
Speight 168 × Red Russian *mlml*, F_1	51.2	14.7	101.1	6323	482.8	322.3	682.2	402.0	628.8	374.8
Speight 168 × Red Russian *MlMl*, F_1	57.5	22.9	152.0	7094	559.2	362.8	655.7	373.2	628.2	358.2
Xanthi	49.5	27.1	89.6	2888	326.6	155.0	352.8	194.5	368.5	206.7
Xanthi × Red Russian *mlml*, F_1	44.3	13.6	78.3	3635	388.0	270.3	522.4	351.6	–	–
Xanthi × Red Russian *MlMl*, F_1	52.8	21.0	120.5	4336	389.7	284.8	492.8	318.3	513.7	328.0
LSD 0.05 (entry × year)	13.0	4.5	43.0	1993	131.9	108.2	85.0	63.3	90.4	60.7
CV % (entry × year)	20.1	19.6	29.3	31	24.0	30.3	11.7	14.5	12.2	14.1

Table 3 Effect of the *Ml* genomic region on six measured traits within the Red Russian genetic background

Trait	*a*	*d*	*d/a*	R^2
Days to flower	5.0	2.4	0.48	0.929
Leaf number	4.5	0.7	0.15	0.993
Plant height (cm)	31.7	16.4	0.52	0.918
Yield (g plot^{-1})	1163.0	917.0	0.79	0.828
Length of third leaf (cm)	55.2	53.8	0.97	0.760
Width of third leaf (cm)	30.9	40.6	1.31	0.635
Length of eighth leaf (cm)	26.2	34.5	1.32	0.634
Width of eighth leaf (cm)	−3.5	16.2	−4.68	0.121

a is the additive value (Edwards et al. 1987) estimated by (*MlM*—*mlml*)/2, positive scores indicates allele with positive effect derived from *N. tomentosa. d* is the dominance value (Edwards et al. 1987) estimated by *Mlml*–(*MlMl* + *mlml*)/2. *d*/*a* is the dominant/additive ratio. R^2 is the amount of variation explained

yield relative to *mlml* hybrids (Table 5). The width of the eigth leaf was significantly lower for *Mlml* genotypes relative to *mlml* genotypes.

Significant negative heterosis was observed for leaf number in F_1 hybrids involving crosses of Red Russian *mlml* with Connecticut Shade 8212, TN 86, and Xanthi (data not shown). Significant heterosis was not observed for this trait in their NIH counterparts involving Red Russian *MlMl*. Significant positive heterosis was detected for plant height in hybrids involving crosses of Red Russian *MlMl* with TN 86 and NC2326. This was not observed for corresponding hybrids involving Red Russian *mlml*. For yield, significant positive heterosis was only observed for the TN86 × Red Russian *mlml* hybrid. The only other cases of significant heterosis were for the lengths and widths of the eigth leaves. For these cases, the degree of heterosis was greater and more significant for hybrids involving Red Russian *mlml* as compared to those involving Red Russian *MlMl*.

In the replicated field study, days to flower, leaf number, plant height, and plot yield were all highly and significantly positively correlated with each other (Table 6). Plot yield was also found to be significantly positively correlated with the lengths of the 3rd, 8th, and 13th leaves, and also the widths of the third and eighth leaves. Most leaf length and width measurements were also found to be significantly positively correlated.

Marker-trait associations

Screening of 160 AFLP primer combinations on DNA isolated from Red Russian *MlMl* and Red Russian *mlml* revealed 15 unambiguous and reproducible markers that were present for the former genotype and absent for the latter (Table 7). These markers were

Table 4 Tests of significance for differences between nearly isogenic hybrids (NIH's) for 10 measured traits

Comparison	Difference[a]									
	Days to flower	Leaf no.	Plant height(cm)	Plot yield (g)	Third leaf length (cm)	Third leaf width (cm)	Eighth leaf length (cm)	Eighth leaf width (cm)	13th leaf length (cm)	13th leaf width (cm)
P. Havana × R. Russian *MlMl* F_1 vs. P. Havana × R. Russian *mlml* F_1	4.0	2.7	17.5	449	13.0	4.7	–	–	–	–
C. Shade 8212 × R. Russian *MlMl* F_1 vs. C. Shade 8212 × R. Russian *mlml* F_1	11.3	9.7***	72.6**	933	20.8	26.8	−16.0	−22.5	17.5	29.6
TN 86 × R. Russian *MlMl* F_1 vs. TN 86 × R. Russian *mlml* F_1	12.7	12.3****	78.1**	220	38.0	22.5	−6.9	−24.2	7.3	30.7
NC2326 × R. Russian *MlMl* F_1 vs. NC2326 × R. Russian *mlml* F_1	9.0	9.1***	58.0*	176	36.8	31.8	−48.1	−65.2**	–	–
Speight 168 × R. Russian *MlMl* F_1 vs. Speight 168 × R. Russian *mlml* F_1	6.3	8.2**	50.9*	771	76.3	40.5	−26.5	−28.8	0.7	16.7
Xanthi × R. Russian *MlMl* F_1 vs. Xanthi × R. Russian *mlml* F_1	8.5	7.4**	42.2	700	1.7	14.5	−29.6	−33.3	–	–

*, **, ***, **** Indicate significant differences at the 0.05, 0.01, 0.001, and <0.0001 levels, respectively

[a] Differences represent *mlml* phenotypic values subtracted from *Mlml* phenotypic values

Table 5 Tests of significance for differences between *Mlml* and *mlml* genotypes over seven genetic backgrounds

Trait	Difference (*Mlml* − *mlml*)
Days to flower	8.5**
Leaf number	7.8****
Plant height (cm)	52.5****
Plot yield (g)	761.5*
Third leaf length (cm)	42.2
Third leaf width (cm)	30.3
Eighth leaf length (cm)	−11.0
Eighth leaf width (cm)	−26.9**
13th leaf length (cm)	−8.5
13th leaf width (cm)	−25.7

*, **, ***, **** Indicate significant differences at the 0.05, 0.01, 0.001, and <0.0001 levels, respectively

subsequently mapped on 207 field-grown BC_1F_1 individuals from the cross Red Russian *MlMl*/Red Russian *mlml*//Red Russian *mlml*, and found to reside in a single linkage group of 34.5 cM (Figs. 1, 2). Nine markers exhibited slight segregation distortion because of lower than expected transmission to female gametes (Table 7). These nine markers all mapped together within a 4 cM region near the end of the linkage group (Figs. 1 and 2). All 15 markers were found to be present in *N. tomentosa* accessions TW 140, ITB 1015, and 91475006. All markers except M12 were found to be present for accession TW 141.

Application of *F*-tests to data collected from the 207 BC_1F_1 individuals indicated significant associations between all markers and days to flower, leaf number, and plant height (data not shown). For markers most significantly associated with these traits, R^2 values were 0.46, 0.61, and 0.47, respectively. Histograms representing phenotypic distributions for these three traits are provided in Fig. 3. Significant associations were also found between most markers and length and width measurements for the fifth and eight leaves. No significant associations were detected between any marker and yield.

Table 7 AFLP markers used to genotype BC_1F_1 individuals, and corresponding Chi-square values for testing for significant deviations from 1:1 segregation ratios

Marker		χ^2
M2	E-AAC/M-CGT-109	1.1
M15	E-AGT/M-CCA-130	0.8
M12	E-ATG/M-CCG-125	1.1
M14	E-AGG/M-CAA-136	1.4
M4	E-ACC/M-CGA-481	1.1
M5	E-ATA/M-CCA-433	1.7
M1	E-ACT/M-CCT-130	6.3*
M10	E-AAC/M-CGA-368	4.3*
M3	E-AAT/M-CGC-595	4.4*
M6	E-AGT/M-CGC-439	4.6*
M7	E-AGT/M-CGC-331	4.6*
M9	E-AGT/M-CCG-410	4.6*
M11	E-AGA/M-CGA-375	4.6*
M8	E-ACT/M-CCC-427	5.3*
M13	E-AGT/M-CAG-289	6.3*

*Indicates deviation from an expected 1:1 ratio at the 0.05 level of significance

The composite interval mapping approach indicated that 10 markers within the introgressed linkage group had LOD scores that exceeded the calculated LOD threshold significance levels (Churchill and Doerge 1994) of 2.28, 2.32, and 2.43 for days to flower, leaf number, and plant height, respectively (Fig. 1). Overlapping 2-LOD confidence intervals (CIs) were identified for a QTL influencing plant height, leaf number, and days to flower. The QTL had 2-LOD CIs of 2.9, 3.1, and 3.3 cM in length, and had LOD peaks of 18.3, 29.2, and 18.7 for the three traits, respectively (Fig. 1). The QTL within the region accounted for 27, 37, and 28% of the observed phenotypic variation for these traits (Table 8), respectively, and was adjacent to AFLP marker M1. BC_1F_1 plants possessing the *N. tomentosa* QTL had delayed flowering (8.1 days), increased leaf number (7.2 leaves), and increased plant height (35.6 cm) (Table 8). No significant QTLs were identified for yield using composite interval mapping (Fig. 1).

Table 6 Pearson correlation coefficients for relationships between characteristics measured on lines and their F_1 hybrids

	Leaf number	Plant height	Plot yield	Third leaf length	Third leaf width	Eighth leaf length	Eighth leaf width	13th leaf length	13th leaf width
Days to flower	0.906****	0.861****	0.829****	0.827****	0.754****	0.494*	0.196	0.256	−0.164
Leaf number		0.831****	0.625**	0.604**	0.498*	0.096	−0.163	−0.256	−0.469
Plant height			0.700***	0.664**	0.718***	0.360	0.368	0.105	0.330
Plot yield				0.954****	0.891****	0.906****	0.602**	0.913****	0.423
Third leaf length					0.904****	0.887****	0.532*	0.819***	0.325
Third leaf Width						0.810****	0.805****	0.727**	0.736**
Eighth leaf length							0.733***	0.959****	0.496
Eighth leaf width								0.666**	0.914****
13th leaf length									0.499

*, **, ***, **** Represent significance at the 0.05, 0.01, 0.001, and <0.0001 levels, respectively

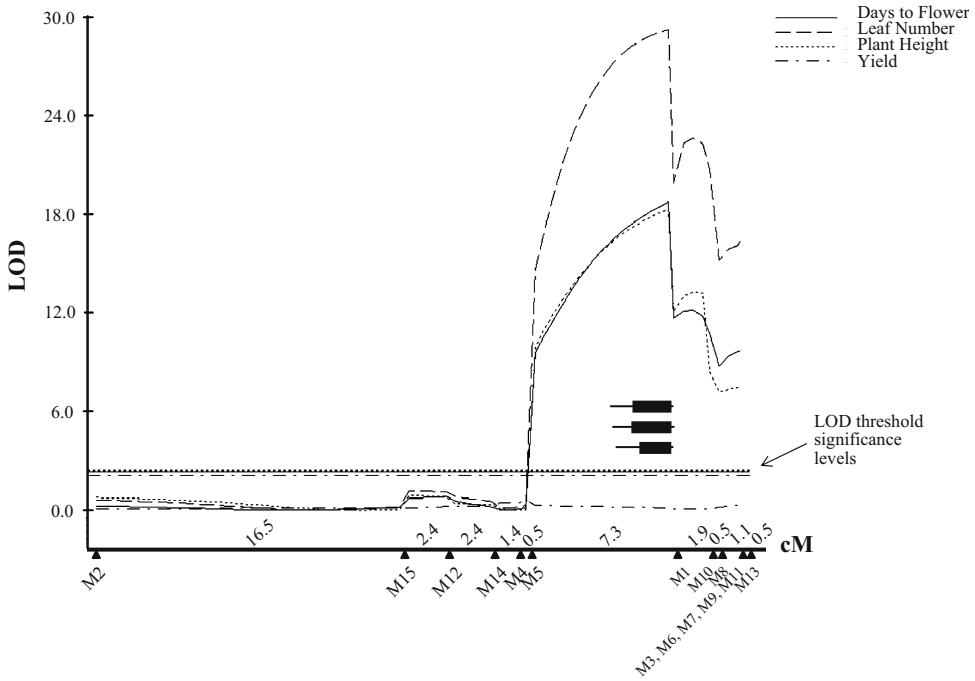

Fig. 1 QTL map for introgressed *N. tomentosa* genomic region produced using composite interval mapping for days to flower, leaf number, plant height, & yield. Markers are indicated beneath the X axis, with intervals between them indicated in cM immediately above the X axis. 2-LOD & 1-LOD confidence intervals for QTL positions are indicated by thin and thickened horizontal bars, respectively, above the X axis (corresponding to plant height, leaf number, & days to flower, from top to bottom). M1 = AFLP marker EACT/MCCT-130, M2 = EAAC/MCGT-109, M3 = EAAT/MCGC-595, M4 = EACC/MCGA-481, M5 = EATA/MCCA-433, M6 = EAGT/MCGC-439, M7 = EAGT/MCGC-331, M8 = EACT/MCCC-427, M9 = EAGT/MCCG-410, M10 = EAAC/MCGA-368, M11 = EAGA/MCGA-375, M12 = EATG/MCCG-125, M13 = MAGT/MCAG-289, M14 = EAGG/MCAA-136, M15 = EAGT/MCCA-130

For leaf measurements, calculated LOD threshold values were exceeded only for the width of the 10th leaf (Fig. 2). Nine contiguous markers exceeded the threshold LOD value of 2.22 for this trait. QTL Cartographer generated a 2-LOD CI of 7.6 cM for a QTL affecting the width of the 10th leaf, and this CI overlapped with those identified for plant height, leaf number, and days to flower. This declared QTL had a LOD peak of 3.52, and explained only 8% of the observed phenotypic variation for this trait (Table 8). BC_1F_1 plants possessing the QTL had an average reduction in the width of the 10th leaf of 34.1 cm.

In the BC_1F_1 population, very significant positive correlations were found for relationships between days to flower, leaf number, and plant height (Table 9). Significant negative correlations were observed for the relationships of days to flower and leaf number with the lengths and widths of the 5th and 10th leaves. Positive significant correlations were observed between measurements for lengths and widths of the 5th and 10th leaves. Plot yield was found to be significantly and positively correlated with plant height, and the lengths and widths of the 5th and 10th leaves.

Discussion

This investigation demonstrates that diploid *Nicotiana* species closely related to *N. tabacum* can contribute alleles with large positive effects on the phenotype of cultivated tobacco. It also demonstrates the potential for sufficient recombination within chromatin introgressed from such species, such that localization of corresponding QTLs may be achieved. This is in contrast to past experiments dealing with chromosome segments carrying disease resistance genes introgressed from *Nicotiana* species more distantly related to *N. tabacum*'s probable progenitor species, *N. tomentosiformis* and *N. sylvestris* (Bai et al. 1995; Johnson et al. 2002; Lewis 2005; Lewis et al. 2005; Milla et al. 2005). Hence, efforts to simultaneously transfer and map useful/interesting genes derived from more closely related species such as *N. otophora* Grisebach, *N. tomentosiformis*, *N. kawakamii* Ohashi, *N. tomentosa*, or *N. sylvestris* may be feasible. Wernsman and Matzinger (1966) previously outlined methods for transferring germplasm from these diploid relatives to the amphidiploid species *N. tabacum*. Previous research has also

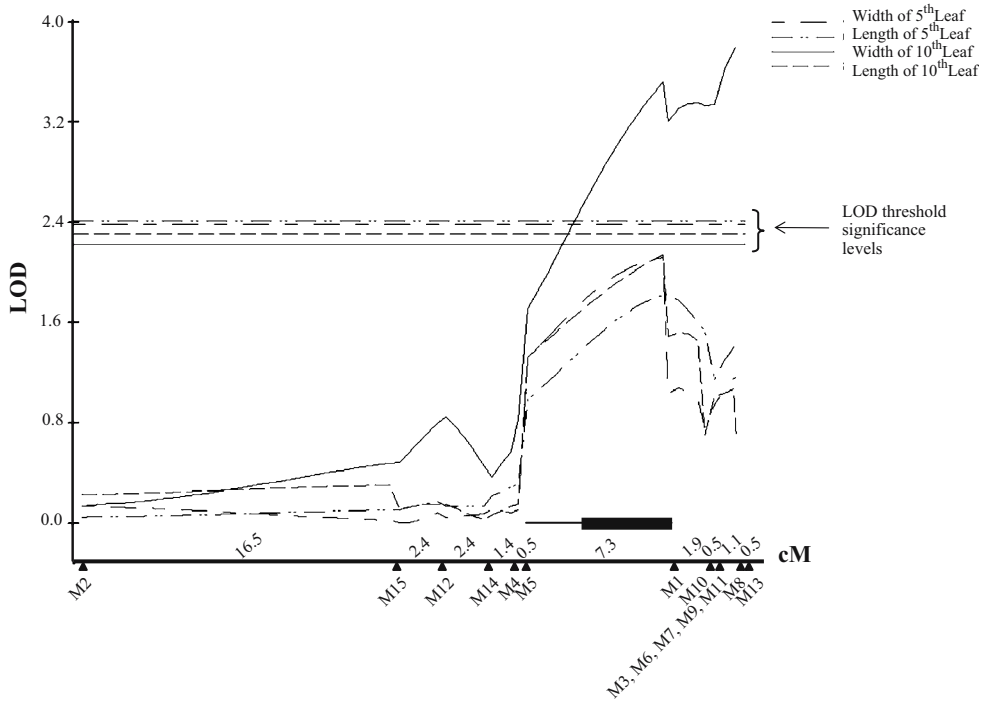

Fig. 2 QTL map for introgressed *N. tomentosa* genomic region produced using composite interval mapping for width of 5th leaf, length of 5th leaf, width of 10th leaf, & length of 10th leaf. Markers are indicated beneath the X axis, with intervals between them indicated in cM immediately above the X axis. 2-LOD & 1-LOD confidence intervals are indicated by thin and thickened horizontal bars, respectively, above the X axis for the position of a likely QTL influencing the width of the 10th leaf. M1 = AFLP marker EACT/MCCT-130, M2 = EAAC/MCGT-109, M3 = EAAT/MCGC-595, M4 = EACC/MCGA-481, M5 = EATA/MCCA-433, M6 = EAGT/MCGC-439, M7 = EAGT/MCGC-331, M8 = EACT/MCCC-427, M9 = EAGT/MCCG-410, M10 = EAAC/MCGA-368, M11 = EAGA/MCGA-375, M12 = EATG/MCCG-125, M13 = MAGT/MCAG-289, M14 = EAGG/MCAA-136, M15 = EAGT/MCCA-130

demonstrated that beneficial alleles likely exist in these species (Wernsman et al. 1976). Exploitation of this diversity has been difficult, however, because of unfavorable associations between alien gene introgression and reduced cured leaf quality (Oupadissakoon and Wernsman 1977). This might be overcome, however, through the use of approaches involving simultaneous favorable QTL identification and precise, marker-assisted transfer to elite genetic backgrounds (Tanksley and Nelson 1996; Huang et al. 2003).

Although several AFLP markers exhibited slight segregation distortion, linkage mapping is robust in the presence of these effects (Hackett and Broadfoot 2003) and we were able to map the likely position of a genetic factor(s) with significant influence on leaf number, flowering time, and plant height within a 3.3 cM region introgressed from *N. tomentosa*. Within a Red Russian genetic background, the *Many Leaves* region was found to act in an additive to partially dominant fashion on days to flower, leaf number, plant height, yield, and the width of the third leaf. Overdominance was observed for the length of the third leaf, and the width and length of the eighth leaf. Further work may provide additional insight on this QTL. In tomato, Eshed and Zamir (1995) subjected NIL regions to fine scale mapping using markers to select for recombinants within these regions. Upon fine mapping, a 37 cM introgressed region possessing factors influencing total soluble solids yield was subdivided by recombination into a partially dominant QTL that increases the trait and a second QTL that decreases the trait when in homozygous condition. Also, a 55 cM region initially identified to influence fruit mass was found to contain at least three separate QTLs.

Many QTL identified in crop plants have been demonstrated to be involved in epistatic relationships (Lark et al. 1995; Eshed and Zamir 1996; Lin et al. 2000). Three types of epistasis might affect complex traits: (1) interactions between QTLs, (2) interactions between QTLs and 'background' (modifying) loci, and (3) interactions between 'complementary' loci. (Li 1998). The development and testing of NIHs differing for the presence/absence of the *Ml* QTL allowed for testing for variability in the effect of *Ml* in different genetic backgrounds. The null hypothesis was that *Ml*

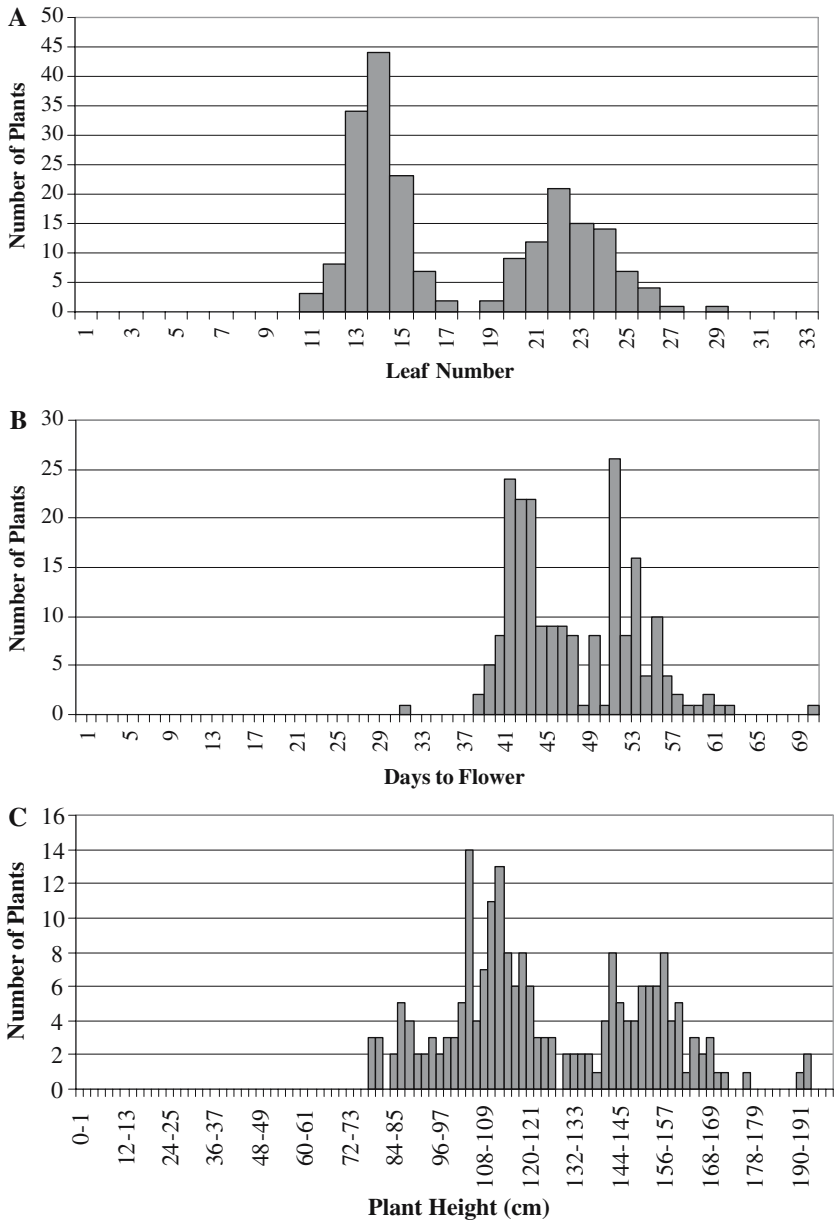

Fig. 3 Phenotypic distributions for 207 BC_1F_1 individuals for (A) leaf number, (B) days to flower, and (C) plant height

Table 8 Biometrical parameters of quantitative trait loci affecting phenotypic variation for four measured traits in a BC_1F_1 population segregating for AFLP markers within an introgressed *N. tomentosa* genomic region

Trait (locus)	Map position (cM)	Effect[a]	R^2	LOD
Days to flower (*M*-a)	30.2	8.11 days	0.28	18.74
Leaf number (*Ml*-b)	30.2	7.17 leaves	0.37	29.24
Plant height (*Ml*-c)	30.2	35.59 cm	0.27	18.30
Width of 10th leaf (*Ml*-d)	30.2	−34.07 cm	0.08	3.52

R^2 indicates the percentage of the total phenotypic variation that is attributed to variation at each locus

[a] Effect is calculated as [marker heterozygote mean (+/−)-marker homozygote mean (−/−)]. A positive value indicates a positive effect of the *N. tomentosa* allele

would have the same effect in all genetic backgrounds (low or high genetic potential for leaf number). The effect of *Ml* on flowering time varied from 4.0 to 12.7 days, and its effect on leaf number varied from 2.7 to 12.3 leaves. It was interesting that, in the materials studied here, *Ml* appeared to have a greater effect in hybrids with increased genetic potential for these traits. This observed result may reflect the importance of epistasis, or modifying factors, on the expression of these traits.

The introgressed chromosome region from *N. tomentosa* exhibited a significant positive influence on leaf number in *N. tabacum*. Modern flue-cured tobacco cultivars are typically topped (decapitated) at 18–20 harvestable leaves plant^{-1}. Tobacco cultivars with genetic

Table 9 Pearson correlation coefficients for relationships between measured traits for BC1F1 individuals

	Leaf number	Plant height	Plot yield	Fifth leaf width	Fifth leaf length	10th leaf width	10th leaf length
Days to flower	0.876****	0.806****	−0.094	−0.345****	−0.324****	−0.411****	−0.280****
Leaf number		0.901****	0.107	−0.285****	−0.248***	−0.323****	−0.206**
Plant height			0.321****	−0.049	−0.003	−0.073	−0.010
Yield				0.660****	0.672****	0.668****	0.617****
Fifth leaf width					0.816****	0.680****	0.585****
Fifth leaf length						0.568****	0.548****
10th leaf width							0.674****

, *, **** represent significance at the 0.01, 0.001, and <0.0001 levels, respectively

potential for increased leaf number can be topped at much higher leaf numbers, as many as 30 leaves plant^{-1}. To date, these cultivars have typically been developed through the use of recessive alleles conferring photoperiod sensitivity and continued vegetative development in temperate regions until the number of hours of light day^{-1} declines to a certain point. Cultivars with increased leaf number offer the potential for increased yields and increased flexibility in crop management (Wernsman and Matzinger 1980; King 1986). In the current investigation, leaf number was highly correlated with yield in the replicated field experiment involving lines and hybrids, but not significantly correlated with yield in the segregating BC_1F_1 population. Yield was significantly higher in lines and hybrids possessing *Ml* as compared to *mlml* lines and hybrids. Although yield was greater in BC_1F_1 plants possessing *N. tomentosa* markers, these differences were not statistically significant. In both the replicated experiment and in the BC_1F_1 population, increases in leaf number came at the expense of decreases in the widths and lengths of leaves at upper stalk positions.

Practices in which cultivars are topped at higher leaf numbers have not been widely adopted in commercial production because of a tendency of these practices to contribute to reductions in profit hectare^{-1} due to reduced cured leaf quality. If cultivars with increased genetic potential for leaf number are to be cultivated at higher leaf numbers in the future, this must be accompanied with new production practices and/or plant genetics that contribute to improved cured leaf quality at these leaf numbers. Genetic factors located within the *Ml* region may be useful as an additional source of variation for leaf number in future tobacco breeding populations.

There is interest in gaining additional insight on the control of the transition of vegetative to reproductive development in plants, including the role of genetic factor(s) within the *Ml* region. NILs have been useful for aiding in the isolation of genes controlling quantitative traits, including those influencing flowering time (Doebley et al. 1995; Frary et al. 2000; Yano et al. 2000; Fridman et al. 2004). Genetic materials and results described in this report may be of value in future experiments to investigate the control of this trait in *Nicotiana*. Future work may include a candidate gene approach (Pflieger et al. 2001) to test whether or not *Nicotiana* sequences orthologous to those previously shown to affect these traits in other species (Yano et al. 2000; El-Din El-Assal et al. 2001; Yan et al. 2004; Abe et al. 2005; Turner et al. 2005; Wigge et al. 2005; Lifschitz et al. 2006) also affect the same characteristics in tobacco populations segregating for *Ml*. Such an approach has been tested in soybeans (Tasma and Shoemaker 2003).

Acknowledgements The authors would like to thank to Philip Morris, USA, for their financial support of the N.C. State University tobacco breeding and genetics research program.

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