Dynamic Changes in the Distribution of a Satellite Homologous to Intergenic 26-18S rDNA Spacer in the Evolution of *Nicotiana*

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ABSTRACT

An ~135-bp sequence called the A1/A2 repeat was isolated from the transcribed region of the 26-18S rDNA intergenic spacer (IGS) of *Nicotiana tomentosiformis*. Fluorescence *in situ* hybridization (FISH) and Southern blot analysis revealed its occurrence as an independent satellite (termed an A1/A2 satellite) outside of rDNA loci in species of *Nicotiana* section *Tomentosae*. The chromosomal location, patterns of genomic dispersion, and copy numbers of its tandemly arranged units varied between the species. In more distantly related *Nicotiana* species the A1/A2 repeats were found only at the nucleolar organizer regions (NOR). There was a trend toward the elimination of the A1/A2 satellite in *N. tabacum* (tobacco), an allotetraploid with parents closely related to the diploids *N. sylvestris* and *N. tomentosiformis*. This process may have already commenced in an S₃ generation of synthetic tobacco. Cytosine residues in the IGS were significantly hypomethylated compared with the A1/A2 satellite. There was no clear separation between the IGS and satellite fractions in sequence analysis of individual clones and we found no evidence for CG suppression. Taken together the data indicate a dynamic nature of the A1/A2 repeats in *Nicotiana* genomes, with evidence for recurrent integration, copy number expansions, and contractions.

PLANT genomes often contain considerable amounts of repetitive sequences. Of these, a few are transcribed, including clusters of ribosomal RNA (rRNA), transfer RNA, and histone genes. The large ribosomal DNA (rDNA) unit cluster (35S rDNA in plants; HEMLEBEN and ZENTGRAF 1994) encoding 18S, 5.8S, and 26S rRNA occurs in one or more chromosomal loci. Variable numbers of rRNA genes (from 1000 to >30,000), forming multigene families in tandem arrays, have been reported for a variety of plant species (for review see HEMLEBEN and ZENTGRAF 1994). The highly conserved genic regions are separated by a more diverged intergenic spacer (IGS), which in turn contains several subrepeated regions. While there is enormous variability in IGS sequences between plant genomes there is usually high homogeneity within a genome. Homogeneity of units is maintained by gene conversion and nonhomologous recombination, forces collectively called concerted evolution (DOVER 1982). Here we characterize a sequence found in the IGS of some *Nicotiana* species that is also scattered as a satellite across the genome.

Satellite sequences usually do not encode structural RNA or protein. Limited transcription has, however, been recently demonstrated and a role for satellite-specific small interfering RNA molecules (siRNA) has been proposed for the establishment of a heterochromatic state (VOLPE *et al.* 2002). In higher plants, satellites may occupy large chromosomal domains, usually at centromeric and subtelomeric positions (SCHMIDT and HESLOP-HARRISON 1998). Even closely related plant species may differ substantially in type and abundance of satellite repeats. It is known that some satellites evolve rapidly while others remain unchanged throughout long evolutionary periods (GREBENSTEIN *et al.* 1995; KING *et al.* 1995; VERSHININ *et al.* 1996; UGAROVIC and PLOHL 2002). Mechanisms leading to the evolution of a novel satellite are not well understood but include integration of exogenous viral DNA (BEJARANO *et al.* 1996), divergence of endogenous viral elements (LANGDON *et al.* 2000), and nonhomologous recombination (SCHWARZACHER *et al.* 1984), processes accompanied by gene conversion, amplification, and translocation (UGAROVIC and PLOHL 2002). In this article we explore possible mechanisms for the origin and evolution of a *Nicotiana* satellite derived from the IGS of rDNA.

In *Nicotiana* several families of repeated sequences have been isolated and characterized by molecular and cytogenetic methods (KOUKALOVA *et al.* 1989; KENTON *et al.* 1993; GAZDOVA *et al.* 1995; CHEN *et al.* 1997; MATYASEK *et al.* 1997; JAKOWITSCH *et al.* 1998; LIM *et al.* 2000b).

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AY397676 and AY397677.

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Some satellites, *e.g.*, the HRS60 family, are structural features of *Nicotiana* chromosomes, and others, *e.g.*, NTRS or GRD, occur in a subgroup of species and appeared more recently in evolution (LIM *et al.* 2000b). LIM *et al.* (2000b) showed that the chromosomal distribution of repetitive sequences could be used to generate a phylogenetic scheme for *Nicotiana* section *Tomentosae*. Molecular cytogenetics provides a useful approach that is independent of those based on intragenic transcribed spacer sequences (CHASE *et al.* 2003) and *matK* (AOKI and ITO 2000; CHASE *et al.* 2003) sequencing. In section *Tomentosae* rDNA clusters occur on homeologous chromosome 3 and in most species also on chromosome 4, indicating a relatively stable chromosomal organization (LIM *et al.* 2000b). However, molecular methods revealed fast evolution of the units at these loci. For example, the length of the IGS varies substantially among *Nicotiana* species (BORISJUK *et al.* 1997) and there is evidence for rapid evolution of IGS sequences in natural (KOVARIK *et al.* 1996; VOLKOV *et al.* 1999; SKALICKÁ *et al.* 2003) and synthetic (SKALICKÁ *et al.* 2003) tobacco. IGS subrepeats probably represent the fastest “molecular clock” in rDNA (HEMLEBEN and ZENTGRAF 1994; FALQUET *et al.* 1997).

We have isolated a 135-bp subrepeated sequence from the IGS of the rDNA unit of *Nicotiana tomentosiformis*. We report the molecular characterization of IGS sequences both within and outside of rDNA loci. We also conduct phylogenetic analyses of these sequences from the two genomic domains and show their chromosomal location in several diploid and allotetraploid *Nicotiana* species.

MATERIALS AND METHODS

Plant material: Natural diploid and tetraploid species are listed in Table 1. The Th37 synthetic tobacco line was derived from one plant generated from ♀ *N. sylvestris* ($2n = 24$) × ♂ *N. tomentosiformis* ($2n = 24$) and converted to a fertile allotetraploid by *in vitro* callus culture (S₀, BURK 1973). Plants were grown in a greenhouse under standard cultivation conditions.

DNA isolation, restriction analysis, and Southern blot hybridization: Total genomic DNA was extracted from a young leaf, using a slightly modified cetylammmonium bromide (CTAB) protocol (SAGHAI-MAROOF *et al.* 1984). DNA was digested with an excess of restriction enzyme (twice for 3 hr) and subjected to electrophoresis on agarose gels. To each lane 1–3 µg of DNA was loaded to detect high- and medium-copy repeats. Following electrophoresis, the ethidium-bromide-stained gels were photographed, blotted onto membranes (Hybond N+, Amersham Pharmacia, Buckinghamshire, UK), and hybridized to [α -³²P]dCTP-labeled DNA probes ($>10^8$ dpm·µg⁻¹ DNA, Dekaprime kit; Fermentas, Vilnius, Lithuania). Oligonucleotide IGS_SR-V probe was labeled by [γ -³²P]ATP in a polynucleotide kinase reaction. Southern hybridization was carried out in 0.25 M Na-phosphate buffer, pH 7.0, supplemented with 7% sodium dodecyl sulfate (SDS) at 65° for 16 hr followed by washing with 2× SSC (1× SSC = 150 mM NaCl, 15 mM Na₃-citrate, pH 7.0), 0.1% SDS (twice for 5 min), 0.2× SSC, and 0.1% SDS (twice for 15 min). Oligonucleotide probe was hybridized at 45° and the blot was

washed with 2× SSC (twice for 15 min). The membranes were exposed to X-ray film (Medix, Hradec Kralove, Czech Republic) for 4–48 hr. A PhosphorImager (Storm; Molecular Dynamics, Sunnyvale, CA) and ImageQuant (Molecular Dynamics) software were used to quantify the hybridization signal.

DNA probes for Southern hybridization: The 18S rDNA probe contained a 1.7-kb *EcoRI* fragment of the 18S rRNA gene subunit from *Solanum lycopersicum* (Kiss *et al.* 1989; accession no. X51576). The 26S rDNA probe was a 220-bp fragment of the 3' end of the tobacco 26S rRNA gene (accession no. X76056) and was obtained by PCR amplification of the region between nucleotide (nt) 2901 (5'-GAATTCACC CAAGTGT TGGGAT-3') and nt 3121 (5'-AGAGGCGTTCAGTCATA ATC-3') with respect to the transcription starting site of the 26S rRNA gene. The IGS_A1/A2 probe was a cloned ~280-bp A1/A2 subrepeat from *N. tomentosiformis* IGS (VOLKOV *et al.* 1999; accession no. Y08427). The IGS_SR-V probe was a 5'-AGGTGTTGAAAGGCACCTCAAGG-3' oligonucleotide between nt 4196 and 4218 (VOLKOV *et al.* 1999; accession no. Y08427).

PCR and cloning procedures: Templates for PCR were prepared as follows: large amounts (50 µg) of total genomic DNA from *N. tomentosiformis* cv. NIC479/84 were digested with an excess of *EcoRV* restriction enzyme and subjected to agarose gel electrophoresis. Material from 12.0- and 4.8-kb fractions was isolated from the agarose gel, using a gel extraction kit (Qiaex II; QIAGEN, Hilden, Germany). PCR amplification was performed with 30–130 ng of genomic DNA as templates (~12.0- and ~4.8-kb fractions, respectively), in a reaction volume of 80 µl containing Taq buffer, MgCl₂ to a final concentration of 1.5 mM, each nucleotide at 0.2 mM, each primer at 0.5 µM, and 1.6 units of thermostable Taq DNA polymerase (DyNAzyme). The PCR was run on a MJ Research (Watertown, MA) PTC100 under the following conditions: 5 min initial denaturation at 94° (hot start); 25 cycles of 30 sec at 94°, 30 sec at 68° (–0.5° per cycle), 30 sec at 72°; 10 cycles of 30 sec at 94°, 30 sec at 55°, 30 sec at 72°; followed by 10 min at 72°. Primers were designed according to the published sequence of *N. tomentosiformis* IGS between 26S and 18S rDNA (accession no. Y08427). Primer sequences for the A2 subrepeat were SubrepA_for 5'-GGTGTGTGTGAGTTGTGTCTGGC-3' and SubrepA_rev 5'-CAATCRAAACRTRTATATRCCCC-3' (Figure 1). PCR generated a ladder of products (~140 to ~700 bp), which were cloned using the QIAGEN PCR cloning kit into the polylinker of pDrive cloning vector (blue/white and ampicillin resistance selection). Representative clones from each IGS and satellite fractions were submitted to the EMBL/GenBank database (AY397676 and AY397677).

DNA sequencing and analysis: Six randomly selected clones of the PCR products (three from 12.0- and three from 4.8-kb fractions, respectively) were purified in QIAGEN Plasmid mini kit columns and sequenced using SP6 and T7 internal oligonucleotide primers. DNA sequencing was performed by automated “cycle sequencing” at the Laboratory of Plant Molecular Physiology, Brno, Czech Republic (ABI PRISM 310 genetic analyzer, Perkin-Elmer, Norwalk, CT). Sequence analyses were performed using the GCG package (version 10.3; Accelrys, San Diego).

Expected values of CpG dinucleotide distributions are calculated from the formula $E(\text{CpG}) = (n - 1) \times f(C) \times f(G)$ where n is the number of bases in the region and $f(N)$ is the frequency of a given nucleotide.

Computer analysis of DNA structure was carried out using CURVATURE software (SHPIGELMAN *et al.* 1993) that implements the nearest-neighbor wedge model of intrinsic DNA curvature.

TABLE 1
List of *Nicotiana* species used in this study

Species	Code no., variety	Source ^a
<i>Nicotiana tomentosiformis</i> Goodsp.	NIC 479/84	G
<i>N. tomentosiformis</i> Goodsp.	TW142	U
<i>N. sylvestris</i> Speg & Comes	—	S
<i>N. tabacum</i> L.	Vielblättriger	B
<i>N. tabacum</i> L.	095 55	K
<i>N. tabacum</i> L.	Petit Havana SR-1	K
<i>N. tabacum</i> L.	Samsun	K
<i>N. tabacum</i> L.	Nee <i>et al.</i> 51789	Bolivia ^b
Synthetic tobacco	Th37 line	U ^c
<i>N. alata</i> Link & Otto	—	B
<i>N. alata</i> Link & Otto	—	K
<i>N. alata</i> Link & Otto	—	B
<i>N. glauca</i> Graham	—	B
<i>N. glutinosa</i> L.	—	K
<i>N. kawakamii</i> Y. Ohashi	—	B
<i>N. longiflora</i> Cav.	NIPA2	K
<i>N. otophora</i> Griseb.	—	K
<i>N. paniculata</i> L.	—	K
<i>N. solanifolia</i> Walp	—	B
<i>N. suaveolens</i> Lehm	—	B
<i>N. tomentosa</i> Ruiz & Pav	—	K
<i>N. undulata</i> Ruiz & Pav	—	K

^a Abbreviations of seedbanks are: B, Bab, district Nitra, Slovakia; G, Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany; K, Royal Botanic Gardens, Kew, United Kingdom; S, SEITA, Institut du Tabac, Bergerac, France; U, U.S. National Plant Germplasm System, U.S. Department of Agriculture (USDA), Agriculture Research Center, North Carolina State University (<http://plants.usda.gov>).

^b La Paz, Bolivia, province of Sud Yungas, 8 km by road north of Huancané on road to Puente Villa, weedy roadside near houses, altitude 2175 m, 16°22.53'S, 67°35.16'W, May 11, 2001; Nee *et al.* 51789 (British Museum; New York Botanical Garden).

^c Synthesized from *N. tomentosiformis* and *N. sylvestris* by L. G. BURK (1973). Seeds were kindly provided by Verne Sisson (USDA, Agriculture Research Center, North Carolina State University). The S₃ generation of plants was used in this study.

Fluorescence *in situ* hybridization: Fluorescence *in situ* hybridization (FISH) was carried out as described in LIM *et al.* (2000b). Two cloned probes were used: (i) the IGS probe, an ~280-bp A1/A2 subrepeat cloned from *N. tomentosiformis* IGS (VOLKOV *et al.* 1999), and (ii) pTa 71, a cloned 9-kb *EcoRI* fragment of the 35S rDNA unit from *Triticum aestivum* (GERLACH and BEDBROOK 1979). These probes were used at a concentration of 4 µg · ml⁻¹ and labeled with digoxigenin-11-dUTP (Roche Biochemicals, Sussex, UK) or biotin-16-dUTP (Sigma Aldrich). In all, the hybridization mix contained 50% (v/v) formamide, 10% (w/v) dextran sulfate, 0.1% (w/v) sodium dodecyl sulfate in 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate). After overnight hybridization at 37°, the slides were washed in 20% (v/v) formamide in 0.1× SSC at 42° at an estimated hybridization stringency of 80–85%. Sites of probe hybridization were detected using 20 µg · ml⁻¹ fluorescein-conjugated anti-digoxigenin IgG (Roche Biochemicals) and 5 µg · ml⁻¹ Cy3-conjugated avidin (Amersham Pharmacia Biotech). Chromosomes were counterstained with 2 µg · ml⁻¹ 4',6-diamidino-2-phenylindole (DAPI) in 4× SSC, mounted in Vectashield medium (Vector Laboratories, Peterborough, UK), and examined using a Leica DM RA2. Images were captured using Openlab (Improvision, Coventry, UK) and assembled to a plate using Adobe Photoshop (Adobe

Systems, Edinburgh). Images were treated for color contrast and brightness uniformly.

RESULTS

Localization of IGS repeats on chromosomes: A 280-bp dimer referred to as A1/A2 subrepeat (VOLKOV *et al.* 1999) was cloned from *N. tomentosiformis* IGS (Figure 1). This was used as a probe in FISH and Southern hybridization experiments. For FISH we conducted simultaneous hybridization of the IGS probe (for A1/A2 units, green fluorescence) and pTa71 (for rDNA genic regions, red fluorescence) to root-tip metaphases. When these probes label a chromosome region independently, the fluorescence color reflects the fluorochrome attached to the probe, but when the probes colocalize, the fluorescence color reflects the ratio of probe label present, leading to yellow and orange fluorescence colors. The experiment revealed that the IGS probe had two distinct distributions: (i) within the rDNA loci in

FIGURE 1.—Restriction enzyme map of the major *N. tomentosiformis* rDNA unit. The positions of probes (thick lines) and restriction fragment lengths are indicated. IGS structural regions (SR) are termed as SR-II (containing C subrepeats), SR-V (downstream from the transcription starting site), and SR-VI (containing A1/A2 subrepeats). Restriction enzymes are as follows: E, *EcoRV*. The A1/A2 subrepeat unit used as a probe is enlarged and contained the following restriction sites: *Bst*NI, *Cla*I, *Scr*FI, *Sfa*NI, *Rsa*I, *Hae*III, and *Dde*I. Positions of PCR primers used for amplification of A1/A2 sequences are indicated by solid arrowheads. Distances are approximately to scale.

locations that were expected from previous karyotype analyses (LIM *et al.* 2000b) and (ii) dispersed in local concentrations on some chromosomes of *N. tomentosiformis* (both NIC 479/84 and TW142 varieties), *N. kawakamii*, *N. tomentosa*, *N. otophora*, and *N. setchellii* (all section *Tomentosae*). The dispersed A1/A2 units were completely absent in *N. glutinosa* (not shown) and *N. sylvestris* (section *Sylvestris*, ex. *Alatae sensu*; KNAPP *et al.* 2004, Figure 3). Sequence analysis shows that *N. glutinosa* should not be in section *Tomentosae* and is more likely a member of section *Undulatae* (CHASE *et al.* 2003; KNAPP *et al.* 2004). There was considerable variability in signal distribution between the species: while in *N. tomentosiformis* the IGS probe hybridized to six or seven chromosomes (at non-rDNA chromosomal loci), in *N.*

setchellii there was only a small amount of signal on the short arm of a small metacentric chromosome. In *N. tomentosa*, the subrepeat hybridized to small metacentrics while in *N. tomentosiformis* the signal was present predominantly on larger chromosomes. Thus there is little apparent phylogenetic signal in the distribution of dispersed A1/A2 subrepeats outside of rDNA loci (A1/A2 satellites) in section *Tomentosae* (Figure 2). In most cases the IGS probe showed a dispersed, speckled signal distribution that often occupied nearly a whole chromosome arm, *e.g.*, in *N. tomentosiformis* chromosomes. Some chromosomes carried more condensed or denser IGS probe signal distribution, particularly in *N. tomentosa* and *N. tomentosiformis* var. NIC479/84. This may indicate both dispersed and clustered organizations

FIGURE 2.—Karyotypes of *Nicotiana* species in section *Tomentosae* probed by FISH, using digoxigenin-labeled (FITC detected, green fluorescence) IGS probe against the A1/A2 repeats and biotin-labeled (Cy3 detected, red fluorescence) pTa71 for 35S rDNA, counterstained with DAPI for DNA (blue fluorescence). Chromosomes are arranged in decreasing order of size except for rDNA carrying chromosomes whose position is identified in LIM *et al.* (2000b). When the IGS repeat and the 35S rDNA probe colocalize, the signal is yellow or orange depending on the relative strength of the two signals. All species have localized concentrations of dispersed A1/A2 satellite sequences. *N. kawakamii* is distinct in that the 35S probe also labels at dispersed locations. Bar, 10 μ m.

FIGURE 3.—FISH experiment labeled as in Figure 2. Metaphase chromosomes are shown. (A–C) *N. sylvestris*. There are three rDNA loci (six sites). The IGS probe colocalizes with the pTa71 at these loci and at no other site. (D) Synthetic *N. tabacum* (Th37.9); (E) labeled chromosomes isolated. The rDNA loci carrying 35S rDNA sequences are found at the terminal end of the short arm of chromosomes T3, S10, S11, and S12 (E, bottom row). The rDNA locus on S12 is amplified (SKALICKA *et al.* 2003); it is also more orange than the other two S-genome loci, appearing more like the T3 locus. There is dispersed IGS signal on the long arm of chromosome T3 and at five other pairs of chromosomes (E, top row). The total IGS signal is less than that in the two cultivars of *N. tomentosiformis* examined. (F) Feral *N. tabacum* collected in Bolivia (accession Nee *et al.* 51789, S. Knapp); (G) labeled chromosomes isolated. There is less dispersed satellite signal than in the synthetic tobacco, its distribution is different (*e.g.*, T3), and the locus on S12 is not amplified. (H) *N. tabacum* cv. 095-55. The abundance of dispersed A1/A2 satellite is lower than that in the feral tobacco and much lower than that in the synthetic tobacco. Bar, 10 μ m.

of the A1/A2 satellite repeats. Interestingly, in *N. kawakamii* the A1/A2 satellite was frequently associated with dispersed rDNA genic units, the latter having been reported previously (LIM *et al.* 2000b).

It would be expected that *N. tabacum* would have an IGS signal distribution that reflected the sum of that found in the diploid progenitors *N. sylvestris* and *N. tomentosiformis*. However, there was reduced A1/A2 satellite signal in all three tobacco lines [synthetic tobacco Th37 (Figure 3, D and E), a feral tobacco (Figure 3, F and G) and cv. 095-55 (Figure 3H)]. The reduction in A1/A2 satellite sequence was least apparent in the synthetic tobacco (Figure 3, D and E) and the sequence distribution on a subset of chromosomes most closely reflected, albeit in reduced abundance, that found in *N. tomentosiformis* (compare labeled chromosomes in

Figure 3E with those in Figure 2). But there are differences; *e.g.*, there is a satellite locus on the long arm of the T3 chromosome of Th37 plants that did not occur on chromosome 3 of any *N. tomentosiformis* varieties studied. The novel locus could have arisen by an allopolyploidy-induced translocation or amplification event.

Distribution of A1/A2 satellite in *Nicotiana* genomes:

We carried out Southern blot hybridization to study the distributions of A1/A2 satellite in the genomes of different *Nicotiana* species. Genomic DNAs were digested with *EcoRV* restriction enzyme, which has a conserved recognition site in the rDNA unit of *Nicotiana* (Figure 1 and BORISJUK *et al.* 1997). Southern blots were hybridized sequentially with the 18S and 26S genic probes and the A1/A2 and SRV IGS probes (Figure

4A). The 18S and 26S probes hybridized to a single (*N. otophora*) or in most cases to multiple (*e.g.*, *N. tomentososa*) fragments. Multiple bands in the <10-kb region indicated the presence of multiple rDNA families and incomplete rDNA homogenization. As expected both IGS probes hybridized to the same fragments as the 18S genic probe. However, an additional band of ~12 kb of variable intensity was revealed in *N. tomentosiformis* (both cultivars), *N. kawakamii*, *N. tomentosa*, *N. setchellii*, and *N. otophora* after hybridization with the A1/A2 probe. The 12-kb signal was nearly (*N. tabacum*) or completely absent in lanes loaded with DNA from *N. glutinosa*, *N. sylvestris*, *N. undulata*, *N. paniculata*, *N. rustica*, *N. alata*, *N. longiflora*, *N. glauca*, *N. solanifolia*, and *N. suaveolens* (Figure 4 and not shown). Since the 12-kb fragment did not hybridize with the 18S, 26S, and SR-V probes it is likely that the band represents a non-rDNA satellite fraction of the IGS-related A1/A2 repeats. The majority of satellite repeats apparently lack a conserved *EcoRV* site although several minor fragments could be visualized after longer exposure of the blot. The complex pattern of hybridization bands in Figure 4B, lane 5, is consistent with extensive rearrangements of the parental IGS in this subline of synthetic tobacco (SKALICKA *et al.* 2003). The distribution of A1/A2 repeats into rDNA and non-rDNA fractions was quantified by counting the radioactivity in the satellite and rDNA fractions after hybridization of the blot with the A1/A2 IGS probe (Figure 4, Table 2). It is evident that the relative abundance of satellite signal in the 12-kb *EcoRV* fraction correlates with FISH analysis (compare Figures 2 and 3 with Figure 4). It is evident that there is a negligible amount of A1/A2 satellite in both cultivated tobacco varieties examined.

Genomic organization of A1/A2 repeats: To study the genomic organization of A1/A2 repeats we isolated two genomic fractions containing IGS and satellite repeats. Genomic DNA from *N. tomentosiformis* var. NIC479/84 was digested with *EcoRV*, gel separated by electrophoresis, and DNAs of 12- and 4.8-kb size fractions were eluted and purified. The purified DNAs, termed as 4.8- and 12-kb *EcoRV* fractions, were subjected to Southern blot analysis using methylation-insensitive restriction enzymes that have recognition sites within the A1/A2 repeats (Figure 5A). Ladder patterns were obtained from

both 12- and 4.8-kb fractions, suggesting that the repeats are tandemly organized in both satellite and IGS fractions. The ladders started at ~140 bp and were slightly irregular in the 4.8-kb *EcoRV* fraction (Figure 5A). In contrast, highly regular long ladders were obtained after digestion of the 12-kb *EcoRV* fraction with *RsaI*, *SfaNI*, and *DdeI*, suggesting that the tandem repeats could form longer arrays of uninterrupted satellite sequences. Another distinction between the two fractions was a prominent ~0.8-kb band present in the 4.8-kb fraction only. Perhaps the ~0.8-kb fragment represents a relatively abundant rDNA family characterized by a 6-unit spacing of neighboring *RsaI* sites.

Relative hypomethylation of IGS sequences has already been described in several plant species (FLAVELL *et al.* 1988; TORRES-RUIZ and HEMLEBEN 1994), including tobacco (B. KOUKALOVA, unpublished data). We were interested in determining the methylation status of IGS repeats within and outside of the rDNA locus. We digested DNA with methylation-sensitive and -insensitive enzymes followed by Southern blot hybridization (Figure 5B). *BstNI* cuts at CCWGG and is methylation insensitive; its nearby *ScoFI* isoschizomere cuts at CCNGG and is sensitive to CNG methylation; *PvuII* cuts at CAGCTG and is sensitive to CNG methylation; *ClaI* cuts at ATC GAT and is sensitive to CG methylation. Methylation-insensitive *BstNI* digested both 4.8- and 12-kb fractions into a series of bands. In contrast, *ScoFI* produced several bands only after digestion of the 4.8-kb fraction, and there was no significant digestion of the 12-kb satellite fraction. Similar results were obtained when *ClaI* and *PvuII* enzymes were used although in these cases no methylation-insensitive isoschizomeres were available to check the presence of recognition sites. Taken together, the data indicate that the A1/A2 repeats within the rDNA, compared with those outside rDNA loci, are relatively hypomethylated at cytosine residues.

Sequencing analysis of rDNA and satellite A1/A2 repeats: To study sequence homology between IGS and satellite repeats we isolated several clones from the purified genomic fractions of *N. tomentosiformis* described previously. We carried out PCR, using oligonucleotide primers (Figure 1) designed according to the published IGS sequence (VOLKOV *et al.* 1999); one primer contained a degenerative sequence to increase the chance

FIGURE 4.—Southern blot hybridization showing the distribution of A1/A2 repeats (A) in *Nicotiana* diploids and (B) in *N. tabacum* and its progenitor species. Genomic DNA was digested with *EcoRV* and hybridized sequentially with the genic and intergenic probes. In A and B the 12-kb fragment visualized by the A1/A2_IGS probe (but not the genic and SR-V_IGS probes) indicated the presence of an rDNA-independent IGS-related satellite (labeled satellite). (B) The A1/A2 probe hybridized relatively weakly to the ~10-kb *EcoRV* band in *N. sylvestris* rDNA (S-rDNA) due to decreased sequence homology between the probe of *N. tomentosiformis* origin and the *N. sylvestris* IGS. The minor bands in the blot (A) that hybridized with the SR-V_IGS probe result from incompletely stripped 26S probe. Lanes 1–4, *N. tabacum* vars. Vielblättriger, Samsun, SR-1, and feral tobacco. Lanes 5 and 6, Th37 synthetic tobacco plants differing in the type of rDNA families. T-rDNA, position of *EcoRV* restriction fragment containing 18S gene linked to the part of IGS of *Tomentosae* origin. S-rDNA, position of monomeric rDNA unit of *N. sylvestris* origin. Asterisks indicate fragments with rearranged IGS units in the synthetic tobacco line (B).

TABLE 2

Relative proportions of A1/A2 copies in the IGS and satellite genomic fractions

Species	% IGS ^a	Satellite
<i>N. glutinosa</i>	>99	<1
<i>N. setchellii</i>	91	9
<i>N. otophora</i>	92	8
<i>N. tomentosa</i>	49	51
<i>N. kawakamii</i>	91	9
<i>N. tomentosiformis</i> var. TW142	67	33
<i>N. tomentosiformis</i> var. NIC479/84	49	51
<i>N. tabacum</i> var. 095 55	>99	<1
<i>N. tabacum</i> var. Vielblättriger	>99	<1
<i>N. tabacum</i> var. SR-1	98	2
<i>N. tabacum</i> var. Samsun	>99	<1
<i>N. tabacum</i> —feral from Bolivia	90	10
Synthetic tobacco—Th37, subline 8	83	17
Synthetic tobacco—Th37, subline 9	74	26

^a The relative proportions (%) of A1/A2 repeats in individual genomic fractions were calculated from hybridization signals in satellite and IGS fractions on the blots in Figure 4. Radioactivity was measured using the rectangle integration method (Image Quant software, Molecular Dynamics) after scanning the blot on a PhosphorImager (Storm, Molecular Dynamics). The formula for calculations is as follows: $IGS = 100 \times A_{IGS} / (A_{IGS} + A_{SAT})$, where IGS is the percentage of A1/A2 repeats in the rDNA fraction, A_{IGS} is radioactivity in the IGS fraction, and A_{SAT} is radioactivity in the satellite fraction. As an example, calculation for *N. setchellii* is shown: $A_{SAT} + A_{IGS} = 8.0 \times 10^5$; $A_{IGS} = 7.3 \times 10^5$; $A_{SAT} = 7.2 \times 10^4$.

of the primer annealing to mutated sites. The PCR products obtained were analyzed by gel electrophoresis. In both samples the products contained fragments of ~140 bp and its multiples, confirming a tandem arrangement of repeats. Amplified DNAs were cloned into the pDrive (QIAGEN) vector. Gel analysis of recombinant clones showed that inserts contained all kinds of oligomers up to pentamers with dimers and trimers being most abundant. Several randomly selected clones from each fraction were sequenced and the data were analyzed with previously obtained sequence (VOLKOV *et al.* 1999). The sequence homology between the clones from each fraction was between 75 and 85%. A Harr-plot analysis revealed a sequence repetition in most inserts, suggesting that these clones harbor more than one complete repeating unit. The units had a conserved length of ~135 bp, and two clones were shorter (~125 bp). The units within and between individual clones were analyzed by the program DISTANCES implemented within the Wisconsin GCG package software. Alignment of repeating units is expressed by a dendrogram in Figure 6. The multiple alignment did not separate satellite and IGS clones into distinct groups, indicating the absence of a sequence unique for particular fractions. The two separate clusters correspond to A1 and A2 versions of the repeat (VOLKOV *et al.* 1999). Units from *N. sylvestris*

IGS fell into the A1 population branch. Most of the clones obtained from PCR amplification (indicated by “p” after the name of a clone) fell into the A2 group as expected from the primer sites (Figure 1). There was no homology between sequenced clones and analogous repeats in potato IGS (STUPAR *et al.* 2002).

Since the A1/A2 repeats are heavily methylated in satellite and to a lesser extent in IGS we investigated whether cytosine methylation has resulted in CG suppression through C to T transitions over a long period of time (GARDINER-GARDEN *et al.* 1992). Analysis of consensus sequences for IGS and satellite monomers shows nearly equal probabilities of CG occurrences. Observed *vs.* expected values were 0.84 for IGS and 1.03 for satellite fractions, suggesting little or no CG depletion. CG-rich IGS subrepeats have also been reported in *Zea* (BUCKLER and HOLTSFORD 1996) and *Cucurbita* (KING *et al.* 1993), but not in several other genomes (UNFRIED *et al.* 1991).

Theoretical analysis modeling natural DNA curvature has revealed that satellite DNA is regularly curved (FITZGERALD *et al.* 1994; FANN *et al.* 2001). The CURVATURE program (SHPIGELMAN *et al.* 1993) was used to analyze the curvature of A1/A2 monomeric units and other *Nicotiana* satellite repeats. In contrast to most other satellites, cloned A1/A2 repeats have relatively straight DNA paths (see supplementary material at <http://www.genetics.org/supplemental/>).

DISCUSSION

We report the isolation and characterization of a repetitive sequence composed of A1/A2 units that occurs (i) as part of the IGS of 26S-18S rDNA in *Nicotiana* and (ii) independently as a high-copy satellite repeat unassociated with rDNA in the genomes of *Nicotiana* section *Tomentosae* (*sensu* KNAPP *et al.* 2004) and tobacco. The A1/A2 subrepeats of the IGS have dispersed into the genome of section *Tomentosae* and were then carried into the tobacco genome upon allopolyploidy involving the diploid progenitors *N. sylvestris* and *N. tomentosiformis*. The absence of A1/A2 satellites outside section *Tomentosae* and tobacco might be caused by a differential capacity of IGS to transpose into other genomic loci and/or by differential genome tolerance toward genomic changes. We now know that the evolution of section *Tomentosae* is associated with the evolution of at least three repetitive sequences, the A1/A2 satellite reported here, NTRS, and GRS characterized previously (GAZDOVA *et al.* 1995; LIM *et al.* 2000b).

The occurrence of IGS-like sequences in plant genomes has been described in plants previously (MAGGINI *et al.* 1991; UNFRIED *et al.* 1991; FALQUET *et al.* 1997; NOUZOVA *et al.* 2001; STUPAR *et al.* 2002), *e.g.*, high homology between the IGS subrepeat and a satellite in *Vigna radiata* (UNFRIED *et al.* 1991) and limited homology in *Phaseolus* (FALQUET *et al.* 1997). Perhaps multiple

FIGURE 5.—Analysis of genomic fractions carrying A1/A2 repeats. (A) Genomic organization studied with methylation-insensitive restriction enzymes; (B) analysis of DNA methylation using methylation-sensitive (*SrfI*, *ClaI*, and *PvuII*) and -insensitive (*BstNI*) restriction enzymes. The rDNA and non-rDNA fractions containing A1/A2 repeats were isolated from the agarose gel after digestion of *N. tomentosiformis* DNA with *EcoRV* and separation of fragments by electrophoresis. The 12-kb *EcoRV* fraction corresponds to the non-rDNA satellite fraction, and the 4.8-kb band to the rDNA fraction. Isolated *EcoRV* fractions were subjected to secondary digestion with enzymes indicated. Note in A the prominent 0.8-kb *RsaI* band and that the enzymes generated ladders of bands. In B the methylation-sensitive enzymes fail to cut the A1/A2 satellite sequences.

types of IGS-related satellites are scattered in some eukaryote genomes (STUPAR *et al.* 2002). These data may indicate different evolutionary timescales of dispersion of the IGS to the genome from the rDNA locus. Indeed, in some cases the sequence divergence allowed discrimination of IGS-related subfamilies (MACAS *et al.* 2003). In *N. tomentosiformis* there is high homology (75–85%) between A1/A2 repeats irrespective of their genomic origin, suggesting perhaps a recent origin (see also lack of CG suppression below).

Properties of A1/A2 units and clusters: The length of the basic A1/A2 units (138 ± 6 bp) is shorter than usual for a satellite repeat (~ 180 bp; VERSHININ and HESLOP-HARRISON 1998) and it lacks typical DNA curvature. Curved DNA and repeat length close to the nucleosomal periodicity have been proposed to facilitate chromatin condensation (FITZGERALD *et al.* 1994; UGARKOVIC and PLOHL 2002). The IGS may have unusual structural features because IGS subrepeats are thought to be involved in the regulation of rDNA expression (HEMLEBEN and ZENTGRAF 1994). But this alone cannot account for their genetic instability being restricted to *Nicotiana* section *Tomentosae* and tobacco.

On the basis of known copy number of rDNA units and number of A1/A2 subrepeats/unit it is estimated that there are $\sim 2 \times 10^4$ copies of A1/A2 satellite repeat units in the *N. tomentosiformis* genome with some variability between the two accessions (Table 2). Despite high homology between A1/A2 satellite units and A1/A2 units in the IGS, Southern analysis did reveal differences in genomic organization associated with epigenetic

modification. First, the A1/A2 satellite contains a larger number of A1/A2 tandem repeats (>20 -mers) than is typical of the IGS. Perhaps there is a limit to the number of subrepeats in the IGS (HEMLEBEN and ZENTGRAF 1994). Second, there were slight irregularities in ladders of IGS fraction, suggesting a more complex arrangement of A1/A2 subrepeats in the IGS. Third, there was relative hypomethylation of cytosine residues in IGS subrepeats. A hypomethylated fraction of the 35S rDNA molecules is frequently correlated with expression (FLAVELL *et al.* 1988; TORRES-RUIZ and HEMLEBEN 1994; CHEN and PIKAARD 1997; CASTILHO *et al.* 1999). On the other hand hypermethylation of A1/A2 satellite repeats is in accord with a transcriptionally silent satellite. Thus the nearly identical repeats may be differentially methylated depending on the locus and genetic environment (KOVARIK *et al.* 2000).

We postulated that since the A1/A2 are differentially methylated, the long-term evolutionary effect may be higher CG suppression in A1/A2 satellite sequences due to spontaneous or enzymatic deamination to TG dinucleotide (GARDINER-GARDEN *et al.* 1992). However, analysis of CG contents in the A1/A2 repeats in satellite and IGS fractions revealed no evidence for CG suppression, and the content of TG/CA is near expectation, assuming a random nucleotide distribution. In contrast, a family of geminiviral sequences (GRD5) and a family of NTRS repeats, each of which integrated into a subset of species in section *Tomentosae* (LIM *et al.* 2000b), do show substantial CG suppression (MATYASEK *et al.* 1997; MURAD *et al.* 2004).

FIGURE 6.—Neighbor-joining dendrogram of rDNA and satellite A1/A2 sequences of *N. tomentosiformis* and *N. sylvestris*. Assignment of clones is as follows: IGS-TOM-5.4p, clone 5, the fourth unit in the row, obtained from PCR cloning of a repeat from intergenic 26-18S rDNA spacer of *N. tomentosiformis*; SAT-TOM-4.4p, clone 4, the fourth unit in the row, obtained from PCR cloning of the satellite repeat from the 12-kb *EcoRV* fraction of *N. tomentosiformis*; IGS-TOM-A1.1, sequence of the subrepeat obtained from cloning of *N. tomentosiformis* IGS in a phage (the last “1” indicates a unit proximal to the transcription starting site); IGS-SYL.1, sequence of the subrepeat obtained from cloning of *N. sylvestris* IGS in a phage (“1” indicates a unit located proximal to the transcription starting site). The sequences of phage clones are taken from VOLKOV *et al.* (1999).

The origin of A1/A2 satellite sequences: The copy number, position, and genomic organization of the A1/A2 satellite is highly variable among closely related species; indeed, differences could be found between lines and accessions of *N. tabacum* and *N. tomentosiformis*, respectively. The variability in distribution of the A1/A2 satellite exceeds the variability of other tandem repeats

previously mapped in *Nicotiana* (LIM *et al.* 2000b). Thus the rate of divergence in the distribution of the A1/A2 satellite exceeds the rate of *Nicotiana* speciation and the overall rate of karyotype divergence. The absence of any sequence distinction between units of the IGS and satellite fraction (Figure 6), coupled with an absence of CG suppression in the satellite fraction despite high overall levels of cytosine methylation, point to a recent origin of the A1/A2 satellite. Yet this satellite fraction is found across all species of *Nicotiana* section *Tomentosae*, which is at odds with a “recent-origin” hypothesis. The most likely explanation for these data is that the A1/A2 satellite has a recent origin arising through repeated *de novo* integrations or sequence evolution via homogenization that influences the IGS and satellite sequences together. We favor the hypothesis that the A1/A2 satellite has arisen repeatedly through evolution of *Tomentosae* possibly by transposition from the IGS region. The dispersed character of FISH signals on many chromosomes may further support this hypothesis.

It is unknown how the A1/A2 units became dispersed across the genome of some *Nicotiana* species. Three possibilities are apparent.

1. Transposition of A1/A2 repeats may be mediated by reverse transcription of primary rRNA transcripts since the IGS A1/A2 subrepeats are located in the external transcribed spacer (ETS) and are removed during rRNA processing (HEMLEBEN and ZENTGRAF 1994; VOLKOV *et al.* 1999). If this occurred, then unit amplification must have occurred at the novel site since Southern blot data (Figure 5A) indicated that the A1/A2 satellite occurs in long simple tandem repeats that are unlikely to be generated by reverse transcription. A subpopulation of A1/A2 repeats displayed a speckle-like FISH pattern on some chromosomes (Figures 2 and 3). These dispersed sites are probably formed by relatively short tandem arrays (Figure 5A) and may represent recent individual integration events. Perhaps insufficient time has elapsed to allow amplification of units to form long arrays.
2. Longer arrays of IGS satellite repeats could be generated directly by an extrachromosomal excision-amplification-reintegration mechanism (STARK *et al.* 1989) recently proposed for evolution of a potato IGS-related satellite (STUPAR *et al.* 2002). Extrachromosomal amplification may be stimulated in tobacco by an *aps* element located upstream of the transcription starting site, which is known to stimulate amplification of linked transgenic DNA (BORISJUK *et al.* 2000). But FISH (Figure 3) and pulsed-field gel electrophoresis (not shown) failed to reveal extrachromosomal IGS sequences.
3. We favor the hypothesis that IGS-related satellite repeats evolved from randomly integrated solitary rDNA units [termed orphones (DE LUCCHINI *et al.*

1988)] by amplification of subrepeats in the intergenic region. Some evidence supports this hypothesis—the genic probe showed faint hybridization signal to several non-NOR loci on *N. kawakamii* chromosomes, suggesting that other sequences in the rDNA units can also show dispersion in this species.

The influence of allopolyploidy on A1/A2 repeats:

Allopolyploidy is often associated with fast genetic change; for example, instability and frequent elimination of a subtelomeric satellite has been observed in most wheat varieties (PESTSOVA *et al.* 1998). Also in newly synthesized Triticum and Aegilops allopolyploids, there is rapid, directed, and reproducible change in the occurrence of molecular markers (OZKAN *et al.* 2001). But data are contradictory since in similar experiments in newly synthesized allopolyploids of Gossypium there was no such change (LIU *et al.* 2001). SKALICKA *et al.* (2003) showed in the fourth generation of synthetic tobacco that there was a rapid change in the A1/A2 repeat structure at rDNA loci of *N. tomentosiformis* origin. Here we observe a lower number of A1/A2 satellite repeats in both synthetic and natural tobacco lines compared to the paternal parent, *N. tomentosiformis*.

There are several explanations:

- i. The A1/A2 satellite is of paternal origin. SONG *et al.* (1995) demonstrated in synthetic allopolyploids of Brassica that molecular markers of paternal origin were preferentially lost and they suggested that there is some instability in the male-derived genome of an allopolyploid in the cytoplasmic background of the female parent [nuclear cytoplasmic interaction (NCI) hypothesis (JIANG and GILL 1994)]. The A1/A2 satellite might have been eliminated as a result of NCI in tobacco.
- ii. In tobacco gene conversion has altered parental rDNA unit structure (VOLKOV *et al.* 1999; LIM *et al.* 2000a). The rearranged rDNA units could have lost the capacity to transpose from the IGS into non-rDNA chromosomal loci. Perhaps a “steady-state” level exists between recurrent recruitment and elimination of sequences. After homogenization of tobacco rDNA to the new unit type, the recruitment of the IGS units might have been compromised. It might not be coincidental that feral tobacco and synthetic Th37 hybrid lines that have retained most of the A1/A2 satellite also showed partial or no homogenization of rDNA units, respectively. Interestingly, rearrangement of the IGS in natural (VOLKOV *et al.* 1999) and synthetic tobacco (SKALICKÁ *et al.* 2003) involved amplification rather than deletion of A1/A2 subrepeats.
- iii. There is a possibility that the expansion of IGS repeats in *N. tomentosiformis* occurred after the divergence of *N. tabacum* (~10,000 years ago; M. W. CHASE, personal communication), rather than after a loss in *N. tabacum* since its formation.

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