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REVIEW

Evolution of rDNA in *Nicotiana* Allopolyploids: A Potential Link between rDNA Homogenization and Epigenetics

ALES KOVARIK[1], MARTINA DADEJOVA[1], YOONG K. LIM[2], MARK W. CHASE[3], JAMES J. CLARKSON[3], SANDRA KNAPP[4] and ANDREW R. LEITCH[2,*]

[1]*Institute of Biophysics, Academy of Sciences of the Czech Republic, CZ-61265 Brno, Czech Republic,* [2]*School of Biological and Chemical Sciences, Queen Mary University of London, London E1 4NS, UK,* [3]*Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3DS, UK and* [4]*Department of Botany, Natural History Museum, London SW7 5BD, UK*

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• *Background* The evolution and biology of rDNA have interested biologists for many years, in part, because of two intriguing processes: (1) nucleolar dominance and (2) sequence homogenization. We review patterns of evolution in rDNA in the angiosperm genus *Nicotiana* to determine consequences of allopolyploidy on these processes.
• *Scope* Allopolyploid species of *Nicotiana* are ideal for studying rDNA evolution because phylogenetic reconstruction of DNA sequences has revealed patterns of species divergence and their parents. From these studies we also know that polyploids formed over widely different timeframes (thousands to millions of years), enabling comparative and temporal studies of rDNA structure, activity and chromosomal distribution. In addition studies on synthetic polyploids enable the consequences of *de novo* polyploidy on rDNA activity to be determined.
• *Conclusions* We propose that rDNA epigenetic expression patterns established even in F_1 hybrids have a material influence on the likely patterns of divergence of rDNA. It is the active rDNA units that are vulnerable to homogenization, which probably acts to reduce mutational load across the active array. Those rDNA units that are epigenetically silenced may be less vulnerable to sequence homogenization. Selection cannot act on these silenced genes, and they are likely to accumulate mutations and eventually be eliminated from the genome. It is likely that whole silenced arrays will be deleted in polyploids of 1 million years of age and older.

Key words: Diploidization, epigenetics, nucleolar dominance, polyploidy, rDNA, ribosomal DNA, sequence homogenization.

INTRODUCTION

In most eukaryotes 5S and 18–5·8–26S nuclear ribosomal DNA (rDNA) units occur in tandem arrays at one or several loci. Each large rDNA unit contains the 18S, 5·8S and 26S rDNA subunits, the internal transcribed spacers (ITS) sequences and the intergenic spacer (IGS). The genes are highly conserved, whereas ITS divergence is sufficient to resolve species relationships within most genera (e.g. *Nicotiana*; Chase *et al.*, 2003). The IGS, which contains the transcription-start site and genetic and epigenetic features that influence the regulation of the downstream genes, diverges more rapidly than ITS. Substantial differences in structure may even occur within a species (Kovarik *et al.*, 2005).

Of particular interest to evolutionary biologists is the pattern of divergence of the whole rDNA array, which is often influenced by sequence homogenization that functions to replace existing genic units with variants of that unit over time, a process known as concerted evolution (Dover *et al.*, 1982; Eickbush and Eickbush, 2007). Concerted evolution itself may be important to maintain sufficient numbers of active rDNA units, and Ohta (1989) suggested from computer models that homogenization acts to reduce mutational load and is favoured by selection. Patterns of ribosomal RNA (rRNA) gene expression are strongly influenced by epigenetic events such as cytosine methylation and histone acetylation, silencing potentially initiated by small interfering RNAs (Preuss and Pikaard, 2007). In hybrid organisms and allopolyploid species (interspecific hybrids with chromosome number duplication) silencing of entire loci is common, often leading to one parental rDNA array being expressed in preference to another, a phenomenon known as nucleolar dominance (Cermeno *et al.*, 1985; Preuss and Pikaard, 2007).

Ribosomal DNA and the nuclear domain of its activity, the nucleolus, have long intrigued biologists; Alberts *et al.* (2002) stated in a 1898 review that there were already 700 references. Despite a huge volume of ongoing research (web-of-science search on 2 July, 2007, for 'rDNA or 'ribosomal DNA' or 'nucleolus' revealed 25 437 hits), there are still critical and fundamental unanswered questions. These include: (*a*) why do many eukaryotes have more rRNA genes than required for ribosome biosynthesis; (*b*) why are large numbers of rDNA units often, but not always, epigenetically silenced, frequently leading to nucleolar dominance; (*c*) why are rDNA evolution and divergence often, but not always, associated with sequence homogenization, leading to concerted evolution; and (*d*) are any or all of these fundamental processes or phenomena interrelated?

* For correspondence. E-mail A.R.Leitch@qmul.ac.uk

This paper aims to review progress made in addressing these questions using polyploid species of the genus *Nicotiana* as a model system. It is not a general review of rDNA, nucleolar dominance or concerted evolution, which have been well reviewed elsewhere (e.g. Coen *et al.*, 1982; Shaw and Jordan, 1995; Pikaard, 1999, 2000; Wendel, 2000; Raska *et al.*, 2006a, b; Volkov *et al.*, 2007). Instead, it reviews patterns of evolution in rDNA in related *Nicotiana* polyploids of different ages, from synthetic allopolyploids that mimic natural species to natural polyploids that formed 4·5 million years ago. Together these data enable us to propose a hypothesis that explains the relationship between nucleolar dominance, concerted evolution and rDNA locus number. We will discuss the implications of predictions of this hypothesis for other polyploid systems.

NATURAL ALLOPOLYPLOIDS OF *NICOTIANA* AND THEIR GENETIC HISTORY

As currently circumscribed, the genus *Nicotiana* includes 76 species distributed in America, Australia and Africa (Knapp *et al.*, 2004). Phylogenetic reconstructions of plastid and nuclear DNA data have established that the genus *Nicotiana* is monophyletic and inform understanding of species relationships, patterns of divergence and likely parents of allopolyploid species (Chase *et al.*, 2003; Clarkson *et al.*, 2004; Knapp *et al.*, 2004). The base chromosome number of the genus is $n = 12$, but more than 30 species (including the entire Australian section *Suaveolentes*) are allopolyploids, although some species in section *Suaveolentes* show secondary chromosome number reductions (chromosome numbers range from 32 to 48) (Goodspeed, 1954; Knapp *et al.*, 2004). Most of the presumed relationships between diploids and polyploids have been confirmed using molecular cytogenetics and mapping of repetitive DNA probes (Kenton *et al.*, 1993; Lim *et al.*, 2000*b*, 2004, 2005, 2006*a*). Table 1 shows the closest living diploid relatives of the progenitors of *Nicotiana* allopolyploid species and sections (see also fig. 1 in Leitch *et al.*, 2008).

To determine the influence of time on the divergence of rDNA and distribution of loci carrying rDNA (nucleolus organizer regions), it is necessary to estimate the time elapsed since the allopolyploids were formed. To do this, Clarkson *et al.* (2004) dated the nodes of a phylogenetic tree that included all diploid taxa, allotetraploid *N. tabacum* and four allotetraploid species of *Nicotiana* section *Repandae*. By combining data sets, they were able to generate trees with good bootstrap support. They determined likely maximum ages of all nodes on the tree (cf. Richardson *et al.*, 2001) using calibrations based on ages of oceanic volcanic islands on which there are endemic species of *Nicotiana*. Section *Repandae* includes four species, two of which (*N. nesophila* and *N. stocktonii*) occur only on the Revillagigedos Islands in the Pacific Ocean (San Benedicto, Socorro and Clarión) far off the coast of Mexico. The oldest of these islands is Clarión, dated at approx. 1·2 million years. Assuming that *N. nesophila* and *N. stocktonii* diverged from mainland taxa subsequent to formation of this island, then the maximum age of the node that separates these species from *N. repanda* on the mainland is approx. 1·2 million years (cf. Clarkson *et al.*, 2004). Likewise, if it is assumed that *N. cordifolia* formed on the island of Masafuera (Juan Fernández Islands off the coast of Chile in the Pacific Ocean), then dating enables predictions of the maximum likely age of the node that separates

TABLE 1. *Likely ages and diploid progenitors of extant* Nicotiana *polyploids*

Allopolyploid	Maternal parent*	Paternal parent†	Maximum likely age (years before present)
N. tabacum	*N. sylvestris*	*N. tomentosiformis*	<200 000
N. rustica	*N. paniculata*	*N. undulata*	<200 000
N. arentsii	*N. undulata*	*N. wigandioides*	<200 000
Nicotiana section *Polydicliae*	*N. obtusifolia*	*N.* section *Petunioides*/ *N. attenuata*‡	~1000 000
Nicotiana section *Repandae*	*N. sylvestris*	*N. obtusifolia*	~4500 000
Nicotiana section *Suaveolentes*	*N. sylvestris*	Unknown	>10 000 000

* Closest living relative of the maternal parent, based on analysis of plastid and nuclear sequence (Clarkson *et al.*, 2004).

† Closest living relative of the paternal parent, based on analysis of plastid and nuclear sequence (Clarkson *et al.*, 2004).

‡ Chase *et al.* (2003) analysed plastid sequence divergence and reported that one of the progenitors was most likely the progenitor of *Nicotiana* section *Petunioides*.

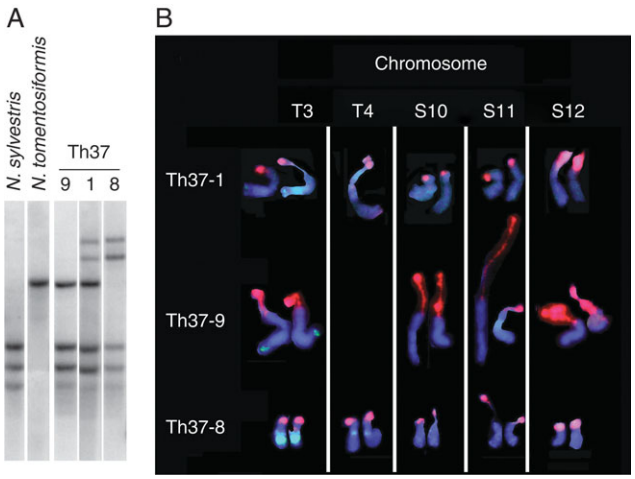

FIG. 1. (A) Southern hybridization showing rDNA polymorphisms in three plants (numbers 1, 8 and 9) of the S_4 generation of Burk's (1973) synthetic *N. tabacum* Th37 line and of the diploid species *N. sylvestris* and *N. tomentosiformis*. Genomic DNA digested with *Bst*NI and probed with 26S rDNA (from Skalicka *et al.*, 2003). (B) FISH showing the number and distribution of 35S rDNA loci (biotin-labelled, Cy3 detected, red fluorescence) to chromosomes [counterstained blue (DAPI) for DNA] in the same Th37 plants (from Skalicka *et al.*, 2003).

N. cordifolia from its sister taxon on the South American mainland, *N. solanifolia*, at 2·4 million years old (Stuessy *et al.*, 1984). Using these as calibration points, it is possible to estimate the maximum age of allopolyploid groups (cf. Table 1; for more details on the dating procedure, see Clarkson *et al.*, 2005).

Estimates of ages for the allopolyploids enable us to examine trends in evolution of rDNA over time. First we explore the consequence of *de novo* polyploidy on rDNA structure, organization and activity by reviewing what is known of synthetic hybrids and allopolyploids, the latter made by interspecific hybridization and chromosome doubling. We compare these data with natural polyploids divided into two categories based on age: young natural allopolyploids (<200 000 years old) and ancient allopolyploids (1–5 million years old).

rDNA AND SYNTHETIC HYBRIDS AND ALLOPOLYPLOIDS

Synthetic hybrids and allopolyploids enable us to follow genetic changes occurring in response to *de novo* allopolyploidy. Any deviation from 'genetic additivity' can be considered to be induced by allopolyploidy. In some cases, *de novo* allopolyploidy is thought to induce a 'genomic shock' responsible for the activation of transposons (McClintock, 1984), retrotransposons (Kashkush *et al.*, 2003; Melayah *et al.*, 2004; Petit *et al.*, 2007), genomic translocations and insertions/deletions (Gill, 1991), and epigenetic reprogramming (Kashkush *et al.*, 2002; Levy and Feldman, 2004; Ozkan *et al.*, 2001). In synthetic allopolyploids of *N. tabacum* (Th37 line, constructed by Burk, 1973), we observed losses of repetitive sequences (including tandem and dispersed repeats and retroelements) predominantly from the paternal genome in early generations (Lim *et al.*, 2004*a*; Skalicka *et al.*, 2005; Petit *et al.*, 2007). Targeted losses of restriction sites [studied via restriction fragment length polymorphism (RFLP) changes], predominantly in the paternal genome, were previously observed in synthetic allopolyploids of *Brassica* (Song *et al.*, 1995). However, not all synthetic allopolyploids show such changes, and there was no change in the profile of amplified fragment length polymorphisms (AFLP) in synthetic allopolyploids of *Gossypium* (Liu *et al.*, 2001).

The first reports of genetic change occurring to rDNA in association with hybrids and allopolyploids in *Nicotiana* were in *N. tabacum* × *Atropa belladonna* hybrids, in which novel rDNA units were observed (Borisjuk *et al.*, 1988). Similarly in an established (1904) horticultural hybrid *N.* × *sanderae* (*N. alata* × *N. forgetiana*; Goodspeed, 1954), new IGS polymorphisms were observed (Lim *et al.*, 2006*a*). In the synthetic tobacco line Th37, only three out of 20 plants analysed showed an additive pattern of rDNA RFLPs observed in the parents. In 75 % of plants, the IGS from *N. tomentosiformis*, accounting for approx. 2000 rDNA units, had been entirely replaced by a novel hybrid-specific rDNA cluster of comparable abundance (Skalicka *et al.*, 2003). All novel rDNA variants were of *N. tomentosiformis* origin but 1–3 kb longer (Fig. 1A), primarily due to amplifications of the SR II and SR VI repetitive subregions of the IGS (subregions first identified by Volkov *et al.*, 1999). Such structural changes in rDNA units have also been encountered in intergeneric somatic hybrids of *N. tabacum* × *A. belladona* (Borisjuk *et al.*, 1988) and interspecific somatic hybrids of *Medicago* (Fabaceae) (Cluster *et al.*, 1996).

We selected three Th37 synthetic tobacco plants with different 35S rDNA RFLP profiles for cytogenetic analysis. (1) Plant Th37·9 had RFLP bands that were additive for those observed in the parents (Fig. 1A) and had an additive number of 35S rDNA loci (i.e. three loci from the maternal parent *N. sylvestris* on chromosomes S10, S11 and S12, and one from the paternal parent *N. tomentosiformis* on chromosome T3; Fig. 1B). (2) Plant Th37·1 had a hybrid-specific cluster of rDNA units (Fig. 1A) and a new rDNA locus on one of the two homologues of chromosome T4 of *N. tomentosiformis*-origin (Fig. 1B). (3) Plant Th37·8 had lost the paternal *N. tomentosiformis*-derived rDNA units, but the rDNA sites on both T3 homologues were still present; the units at these sites had been replaced by the new hybrid-specific cluster of rDNA units (Fig. 1A). In addition both homologues of chromosome T4 now carried a new site (Lim *et al.*, 2000*a*).

Analysis of Th37 synthetic *N. tabacum* (Skalicka *et al.*, 2003) revealed that: (*a*) in most plants there was a rapid amplification of 35S rDNA units (in four generations) involving many thousands of genes that were either new or in subthreshold copy numbers for detection in *N. tomentosiformis*; (*b*) amplification of 35S rDNA units first occurred at a new chromosomal locus (on chromosome T4; similar amplification of units at a new locus has also been observed in synthetic allopolyploids of *Arabidopsis suecica*; Pontes *et al.*, 2004); and (*c*) new 35S rDNA units in most plants had been transferred to chromosome T3. The mechanism for this last observation could have been (*a*) sequence homogenization, an ill-defined process involving recombination machinery and perhaps unequal recombination between homologous or non-homologous sequences, saltatory replication and rDNA unit amplification or (*b*) reciprocal translocations between the rDNA loci on chromosomes T3 and T4, followed by random segregation of chromosomes at meiosis that could lead to the loss of all chromosome T3-derived rDNA units in subsequent generations.

F_1 *N. sylvestris* × *N. tomentosiformis* hybrids (Lim *et al.*, 2006*b*) showed unaltered additivity even after duplication of chromosomes, suggesting that interspecific hybridization and chromosome duplication do not always lead to genetic changes. Similar observations were reported by Dadejova *et al.* (2007) in an analysis of two F_1 hybrids, *N. paniculata* × *N. undulata* (synthetic *N. rustica*) and *N. undulata* × *N. wigandioides* (synthetic *N. arentsii*). Perhaps one or more meiotic cycles are needed to promote rapid and extensive reorganization of 35S rDNA units, copy numbers and/or chromosomal distributions.

rDNA AND YOUNG NATURAL ALLOPOLYPLOIDS (<200 000 YEARS OLD)

Three natural allopolyploids are thought to have formed within the last 200 000 years: *N. tabacum*, *N. rustica* and

N. arentsii. The closest living descendents of the parents are shown in Table 1. Cytogenetic analyses using genomic *in situ* hybridization (GISH) revealed that each polyploid is $2n = 4x = 48$, with 24 chromosomes from each diploid parent (Lim *et al.*, 2004*a*, 2005). The chromosomes also carry satellite repeats observed in the parental species, and only *N. tabacum* exhibits intergenomic translocations, some of which are in all cultivars and some cultivar specific (Lim *et al.*, 2004*a*, 2005). All species also have the sum of the rDNA loci expected from the numbers observed in the parents (Fig. 2). However, the allopolyploids have a decrease in rDNA copy number relative to expectation (based on measurements of rDNA copy numbers from one accession of each parental species) (Lim *et al.*, 2000*a*; Dadejova *et al.*, 2007). If there had been heteromorphy in the number of 35S rDNA loci in the early ancestors of these species, as is observed in synthetic *N. tabacum* (see section 'rDNA in synthetic hybrids and allopolyploids'), it has been lost in modern populations (Kovarik *et al.*, 2004). Likewise, the novel rDNA loci reported in synthetic *A. suecica* are not observed in natural *A. suecica* (Pontes *et al.*, 2004). Thus, these synthetic allopolyploids display greater variation in rDNA locus numbers than their natural counterparts. Perhaps early population bottlenecks purged much of the genetic variation induced by *de novo* allopolyploidy.

RFLP analysis of the structure of the 35S rDNA units in *N. tabacum* revealed that the pattern of bands does not correspond to that found in extant *N. tomentosiformis* and *N. sylvestris* (Kovarik *et al.*, 1997; Lim *et al.*, 2000*a*), suggesting that *N. tabacum* has evolved its own distinct gene family. Sequence analysis revealed that the tobacco-specific units arose by reorganization of the parental *N. tomentosiformis*-inherited units followed by their subsequent amplification and homogenization between rDNA loci (Volkov *et al.*, 1999). The sequence changes mainly involved amplification and reduction of subrepeats upstream and downstream of transcription start sites whereas point mutations were found to be rare.

Similarly, *N. rustica* and *N. arentsii* have also lost parental units (Fig. 3), although retaining the expected number of rDNA loci. However, the extent of parental gene replacement varied significantly between species. The extent of rDNA sequence homogenization decreases in the order *N. arentsii* > *N. tabacum* > *N. rustica*. *Nicotiana rustica* still maintains a considerable number of unconverted *N. paniculata*-origin units. In both *N. tabacum* and *N. rustica* upstream IGS subrepeats were fully homogenized, whereas downstream subrepeats were only partly homogenized, enabling IGS to be resolved into several distinctive families (Matyasek *et al.*, 2003; Kovarik *et al.*, 2004). IGS subrepeats may have a role in homogenization, perhaps by promoting or facilitating recombination between units. In *Nicotiana*, shorter upstream SRII subrepeats (10–23 bp) could undergo recombination more rapidly than the longer SRVI subrepeats (approx. 135 bp), or perhaps they diverge more rapidly. In *Drosophila melanogaster* the IGS contains topoisomerase II sites that are thought to promote recombination and homogenization. The putative AT-rich topoisomerase II binding element has been cloned from *N. tomentosiformis* IGS and shown to promote amplification of a linked reporter transgene (Borisjuk *et al.*, 2000).

All *N. tabacum* cultivars show relatively uniform RFLP profiles, with >90 % of novel hybrid-specific units and <10 % of unconverted *N. sylvestris*-types of unit (Skalicka *et al.*, 2003). However, a feral tobacco collected in Bolivia had a higher proportion of *N. sylvestris*-type unit (20 %), indicating that cultivation and breeding may accelerate the action of homogenization (Kovarik *et al.*, 2004) or that cultivated strains were derived from a narrow range of genotypes compared with variation exhibited by plants in South America (truly wild plants of tobacco are unknown – they are always associated with human habitation); certainly we can expect inbreeding to promote a reduction in heterozygosity and fixation of alleles. Dadejova *et al.* (2007) examined seven varieties of cultivated *N. rustica*, which contained rDNA units designated as P- and U-units depending on the ancestry from the *N. paniculata* or *N. undulata* progenitor (Fig. 3B). All varieties displayed a reduced number of *N. paniculata*-origin units relative to prediction. However, the proportion of

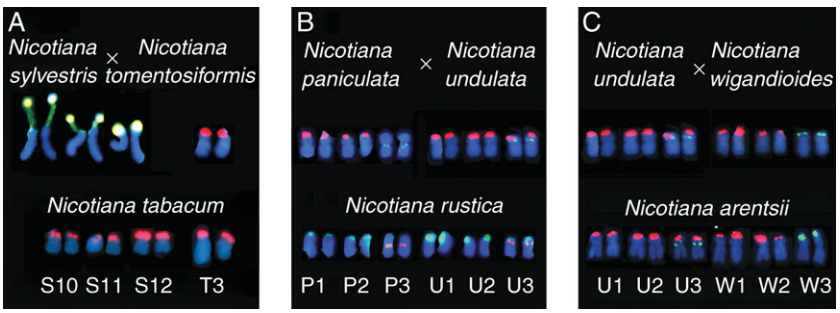

FIG. 2. FISH showing the number and chromosomal distribution of rDNA. (A) *Nicotiana sylvestris* showing 35S rDNA (yellow fluorescence, from Dadejova *et al.*, 2007); *N. tomentosiformis* showing 35S rDNA (red fluorescence, from Dadejova *et al.*, 2007); *N. tabacum* showing 35S rDNA (red fluorescence, new data, biotin labelled 35S rDNA probe, detected with Cy3 fluorescence using methods in Dadejova *et al.*, 2007). (B) *Nicotiana paniculata* and *N. undulata* showing 35S (red fluorescence) and 5S (green fluorescence) rDNA, both from Dadejova *et al.* (2007); *N. rustica* showing 35S (green fluorescence) and 5S (red fluorescence) rDNA (from Lim *et al.*, 2005). (C) *Nicotiana undulata*, *N. wigandioides* and *N. arentsii* showing 35S (red fluorescence) and 5S (green fluorescence) rDNA [from Dadejova *et al.* (2007) and Lim *et al.* (2005)].

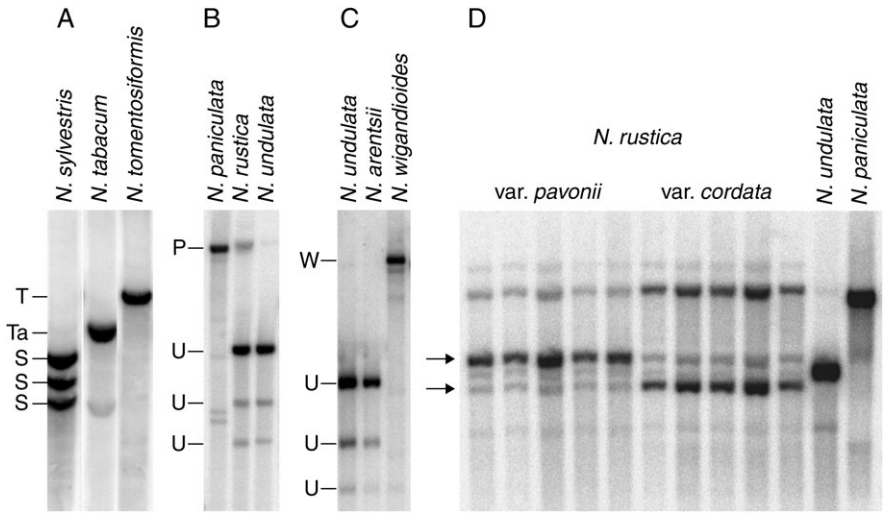

FIG. 3. Southern hybridization showing rDNA polymorphisms in polyploid species *N. tabacum* (A), *N. rustica* (B, D) and *N. arentsii* (C) with those from the diploids most closely related to the parents of the allopolyploids. Genomic DNA digested with (A) *Bst*NI and probed with 26S rDNA (Lim *et al.*, 2000*a*); (B) *Eco*RV and probed with 18S rDNA (Matyasek *et al.*, 2003); (C) *Eco*RV and probed with 18S rDNA (Kovarik *et al.*, 2004). (D) *Eco*RV and probed with 26S rDNA (new data using methods described in Dadejova *et al.*, 2007). Ribosomal DNA units characteristic of a species are indicated: T = *N. tomentosiformis*, Ta = *N. tabacum*, S = *N. sylvestris*, P = *N. paniculata*, U = *N. undulata*, W = *N. wigandioides*. In (D) arrows indicate variety-specific bands corresponding to a major gene family derived from *N. undulata*. Note that the parental IGS types (U and P) are under-represented in a hybrid.

N. paniculata-origin rDNA varied (10–40 %) between, but not within, varieties (Fig. 3D). In addition, analysis of the IGS subregion downstream from the 26S gene showed significant length polymorphism between varieties, probably reflecting IGS rearrangements and further homogenization in the course of variety diversification. Such IGS polymorphisms in crop plants can be potentially exploited for genotyping purposes in breeding programmes.

It may be surprising that molecular reorganization of rDNA units and their amplification within and between rDNA loci are more rapid genetic events than changes in numbers of rDNA loci. *Nicotiana* is not exceptional; allopolyploids of *Gossypium* that are several million years old have also maintained the expected rDNA locus number, whereas the parental units at these loci have been overwritten with new, variant sequences (Wendel *et al.*, 1995). However, over millions of years of allopolyploid divergence in *Nicotiana*, we observe a reduction in rDNA locus number. Thus in cotton, locus number may be a more conserved genetic trait than in *Nicotiana* (see below). Variation in locus number also seems to accompany evolution of many diploid (Dubcovsky and Dvorak, 1995; Lim *et al.*, 2000*b*, 2007; Siroky *et al.*, 2001; Dobigny *et al.*, 2003) and even autopolyploid species (Weiss-Schneeweiss *et al.*, 2007). No apparent reduction of locus number was observed in *Brassica* (Hasterok *et al.*, 2006), recent *Nicotiana* (Kenton *et al.*, 1993; Moscone *et al.*, 1996; Lim *et al.*, 2000*a*; Matyasek *et al.*, 2003) and *Tragopogon* (Kovarik *et al.*, 2005) allopolyploids. These studies indicated that locus number changes are not necessarily associated with the early evolution of allopolyploids.

The presence of activity at any particular rDNA locus, giving rise to nucleolar dominance, can be influenced genetically by the action of homogenization (termed here genetic rDNA dominance) or epigenetically by, for example, cytosine methylation and histone acetylation (termed here epigenetic rDNA dominance). Homogenization of rDNA arrays is probably an ongoing process in many eukaryotes and may function to maintain a high proportion of functional rDNA units (Ohta, 1989). It also leads to the divergence of the IGS, which is not under such strong selective constraints. Nevertheless, proteins that bind to the IGS, including those that function in the regulation of the rDNA unit, must presumably co-evolve with the changing structure of the IGS.

rDNA AND OLD ALLOPOLYPLOIDS (APPROX. 1–5 MILLION YEARS OLD)

Two allopolyploid sections in *Nicotiana* are considered here: (1) section *Polydicliae*, comprising *N. clevelandii* and *N. quadrivalvis*, estimated to have formed approx. 1 million years ago (from progenitors of diploid *N. obtusifolia* + *N. attenuata*); and (2) section *Repandae*, comprising *N. nesophila*, *N. repanda*, *N. stocktonii* and *N. nudicaulis*, estimated to have formed approx. 4·5 million years ago (from progenitors of diploid *N. sylvestris* + *N. obtusifolia*) (Clarkson *et al.*, 2005; Lim *et al.*, 2007). All these polyploids have the expected chromosome number ($2n = 4x = 48$). GISH to *N. quadrivalvis* is effective in that two parental genomes can be resolved using *N. obtusifolia* and *N. attenuata* total genomic DNAs as probes (Lim *et al.*, 2007). However, there is considerable mixing of probe labels between genomes, indicating substantial mobility of DNA between chromosome sets after about 1 million years of divergence. Homogenization of retroelements between subgenomes is also revealed over similar time frames in analyses of *Gossypium* allopolyploids (Zhao *et al.*, 1998). In contrast,

GISH to *N. nesophila* failed, with little DNA labelling by probes of *N. sylvestris* and *N. obtusifolia*-origin chromosomes. These data led us to consider that the majority of the repetitive fraction of the genome has been 'turned over' and replaced by new or variant sequences over a time frame of approx. 5 million years (Lim *et al.*, 2007).

An analysis of the number of 5S and 35S rDNA loci in species of sections *Polydicliae* (Fig. 4) and *Repandae* (Fig. 5) revealed a marked reduction in number from expectation. This reduction results in the total number of sites typical of *Nicotiana* diploids (i.e. two or three 35S rDNA sites and one 5S rDNA site). These allopolyploid species in sections *Polydicliae* and *Repandae* also show unique rDNA RFLPs that differ from diploid parents, indicating that each species has evolved unique rDNA family/ies. *Nicotiana nesophila*, *N. repanda* and *N. stocktonii* possess similar ITS sequences, suggesting their recent diversification from a common allopolyploid ancestor (Chase *et al.*, 2003). However, each species has unique IGSs (Clarkson *et al.*, 2005), indicating either that (*a*) homogenization of IGS occurs more rapidly than that of ITS, or (*b*) homogenization is equally rapid, but changes to ITS are selected against more strongly than those to the IGS.

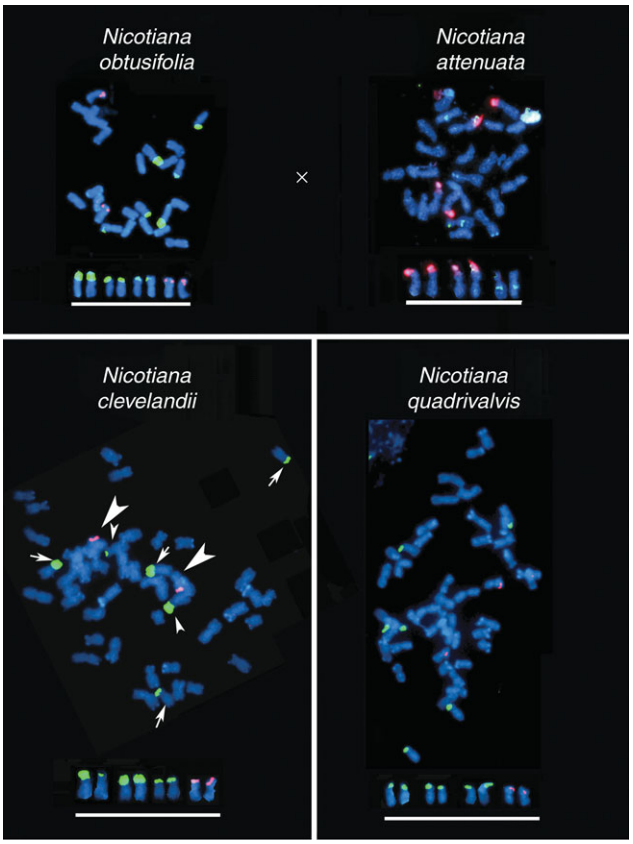

FIG. 4. FISH showing the number and distribution of rDNA loci in diploid progenitors (*N. obtusifolia* and *N. attenuata*) and derived allopolyploids (*N. clevelandii* and *N. quadrivalvis*): *N. obtusifolia*, *N. clevelandii* and *N. quadrivalvis* showing 35S (yellow fluorescence) and 5S (red fluorescence) rDNA. *N. attenuata* showing 35S (red fluorescence) and 5S rDNA (yellow fluorescence). Data from *N. obtusifolia* were taken from Clarkson *et al.* (2005), the remaining data are new [digoxigenin-labelled probes were detected by FITC fluorescence (yellow); biotin-labelled probes with Cy3 fluorescence (red)]. Probes and methods are described in Clarkson *et al.* (2005). Scale bar = 10 μm (metaphases).

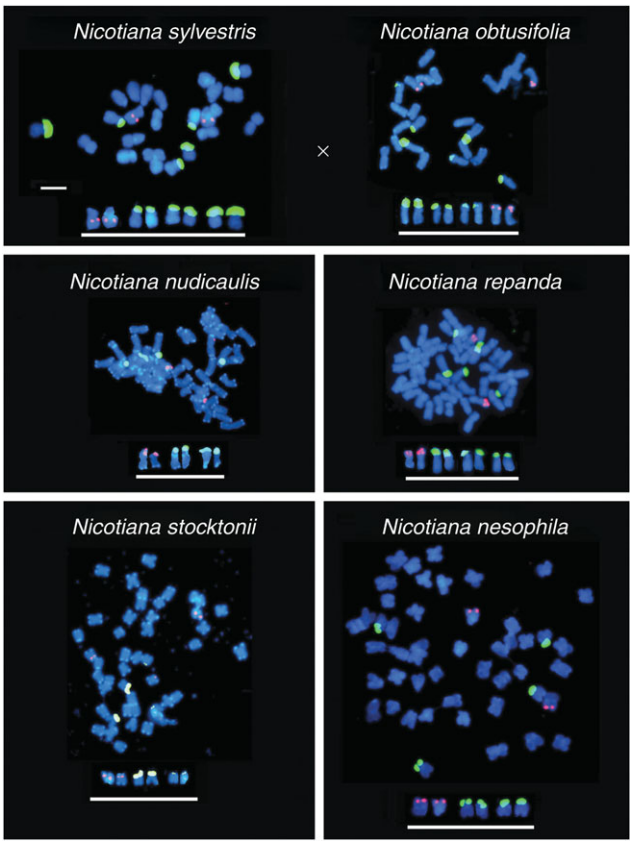

FIG. 5. FISH showing the numbers and distributions of 35S (yellow fluorescence) and 5S (red fluorescence) rDNA loci. Data for the diploid progenitor species *N. sylvestris* and *N. obtusifolia* are taken from Clarkson *et al.* (2005) while those from the derived allopolyploid species (*N. nudicaulis*, *N. repanda*, *N. stocktonii* and *N. nesophila*) are new (using methods described in Clarkson *et al.*, 2005). Scale bar = 10 μm (metaphases).

EPIGENETIC SILENCING AND SEQUENCE HOMOGENIZATION

Epigenetic silencing of rRNA genes, known as nucleolar dominance, has been known for >70 years (Navashin, 1934). The consequence of nucleolar dominance in many polyploids is that one parental gene family is transcriptionally silenced. Previous cytogenetic data in *N. tabacum* indicated that unconverted parental *N. sylvestris*-origin units were highly methylated, perhaps located at a locus on chromosome S12 (Fig. 2A) (Lim *et al.*, 2000*a*) that does not show secondary constrictions at metaphase (a hallmark of genetic inactivity). More recently Dadejova *et al.* (2007) used RT-PCR to investigate expression of rRNA genes in *N. rustica* and *N. tabacum* and observed that units that were not replaced by gene conversion were transcriptionally silenced (i.e. epigenetic rDNA dominance). These authors also showed that there was strong uniparental rDNA silencing in *N. paniculata* × *N. undulata* F_1 hybrids, whereas *N. sylvestris* × *N. tomentosiformis* and *N. undulata* ×

N. wigandioides F_1 hybrids showed little or no rRNA gene silencing (i.e. co-dominance). Occurrence of both nucleolar dominance and locus co-dominance has been described elsewhere, e.g. in polyploids and hybrids involving wheat (Lacadena *et al.*, 1984; Cermeno *et al.*, 1985).

We propose that nucleolar dominance, established early in allopolyploid formation including F_1 hybrids, plays a significant role in the evolution of rDNA. We suggest that epigenetic silencing of rDNA loci makes them less vulnerable to homogenization and more likely to be lost, perhaps thousands or millions of years later. The data from *Nicotiana* point to a link between epigenetic (nucleolar dominance) factors and genetic outcome (homogenization).

(1) In *N. tabacum* and *N. rustica*, rDNA units that are unconverted and inherited little changed genetically from the diploid progenitor are epigenetically silenced. Those units that are active are newly formed hybrid-specific units and carry a clear signature of homogenization in their ancestry (cf. Kovarik *et al.*, 2004).
(2) Unconverted rDNA units in natural polyploids occur outside the nucleolus and contain heterochromatin (Lim *et al* 2000*a*; Matyasek *et al.*, 2003).
(3) There is an inverse correlation between the degree of epigenetic silencing of parental rDNA units in F_1 hybrids and the proportion of parental units that have been homogenized in natural allopolyploids (Fig. 6).

In early allopolyploids there may be over-expression of genes or imbalanced gene-product dosages arising from genome duplication. If this applies to rDNA, there are several ways that gene expression patterns can be tuned to meet cellular/organism physiology: (*a*) physical elimination of genes; (*b*) epigenetic inactivation of units (epigenetic rDNA dominance); (*c*) overwriting of one rDNA variant with another – perhaps by a version that will be silenced (genetic rDNA dominance); or (4) a combination of these. Active rRNA genes organized as decondensed chromatin may be most vulnerable to recombination events that can promote both homogenization and altered copy numbers (Kobayashi and Ganley, 2005; Ganley and Kobayashi, 2007). As a consequence, hybrid-specific units may evolve relatively rapidly, colonizing nucleolus organizer regions at all active loci, potentially from both subgenomes of an allopolyploid (depending on whether both are active). Epigenetic inactivation will increase levels of chromatin condensation and heterochromatin, which might act to protect rDNA from genetic recombination. Under this scenario, inactive, non-homogenized rDNA parental units may be transmitted over many generations even in high copy numbers. Recent findings indicate that rDNA may become dispersed in yeast mutants with abnormal methylation of histone proteins, production of siRNAs and factors involved in chromatin condensation (Peng and Karpen, 2007) showing linkage between genetic stability of repeats and epigenetic pathways. In the future it will be worth analysing epigenetic markers of chromatin (e.g. histone H3K9 methylation) that have survived unaltered during homogenization in allopolyploid species.

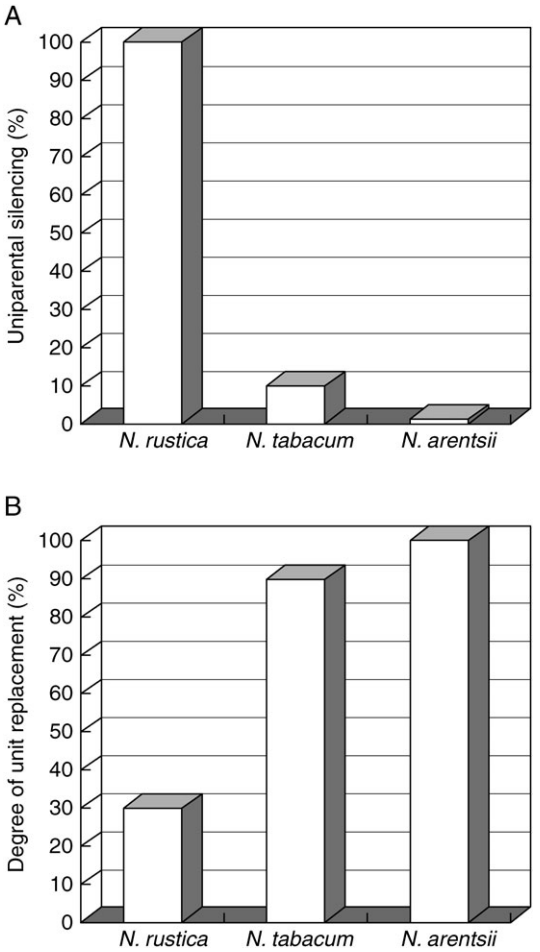

FIG. 6. The relationship between epigenetic silencing in synthetic F_1 hybrids and retention of parental rRNA genes in natural allopolyploids. Data and further details from Dadejova *et al.* (2007). (A) Uniparental silencing of rRNA genes in F_1 hybrids made from the diploid species that most closely resemble the parents of the allopolyploids *N. rustica*, *N. tabacum* and *N. arentsii*. The percentage of silencing was calculated from the ratio of abundance of each parental rRNA transcript; zero indicates no silencing (codominance), 100 % indicates complete silencing of one parental homoeologue. (B) The proportion of rRNA genes that are replaced with alternative units by intergenomic homogenization (i.e. between parental loci) in natural polyploids. Zero indicates no intergenomic homogenization; 100 % indicates complete intergenomic homogenization. The replacement of parental units by intragenomic homogenization (within the locus) has not been considered. Note: a novel major repeat type based on rearrangements of the IGS has evolved in all allopolyploids (Fig. 3).

Homogenization is a powerful process that keeps the tandem arrays uniform, acting to remove non-functional units from the cluster (Ohta, 1989). We can anticipate that silenced rDNA units that are not homogenized would acquire changes leading to loss of function and could potentially be lost over evolutionary time. Certainly no alteration of inactive units would have any selective consequence. Thus, we can predict that the consequences of epigenetic silencing of rDNA loci are: (*a*) reduced or eliminated interlocus homogenization (and perhaps also intralocus homogenization); (*b*) divergence of units in the silenced loci, including substitutions that lead to loss of function, indels and loss of array

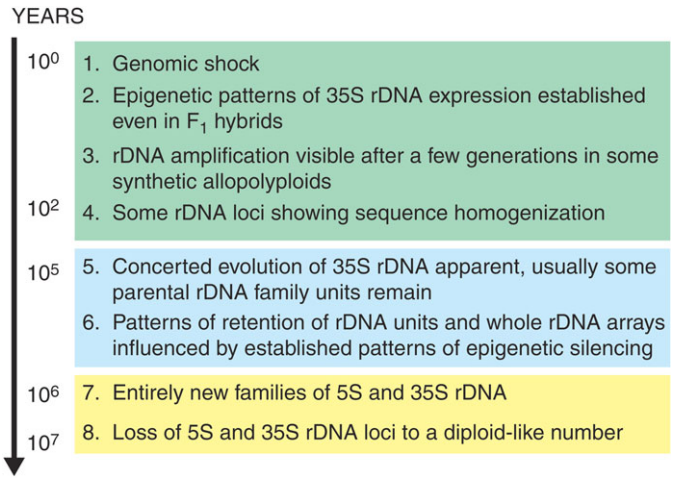

FIG. 7. Summary of rDNA evolution after the formation of *Nicotiana* allopolyploids. The background colours separate allopolyploids according to categories established here: green background, *de novo* polyploids (from synthetic polyploids, few generations); blue background, young polyploids (<200 000 years old); yellow background, old polyploids (1–5 million years old).

uniformity; and (*c*) deletion of silenced genes (Fig. 7), perhaps through accumulation of indels, as for ancient retroelements (Ma *et al.*, 2004), or, in those species in which rDNA arrays occupy a subterminal position, via deletions of chromosome termini. If this cascade of events does apply, then we might envisage that the lost rDNA loci in sections *Repandae* and *Polydicliae* were epigenetically silenced early in evolution of allopolyploid sections.

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