Heredity (2003) 91, 268–275

www.nature.com/hdy

Ribosomal DNA evolution and gene conversion in *Nicotiana rustica*

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Genomic *in situ* hybridisation was used to confirm that *Nicotiana rustica* ($2n=4x=48$) is an allotetraploid between *N. paniculata* ($2n=2x=24$, maternal P- genome donor) and *N. undulata* ($2n=2x=24$, paternal U-genome donor), their progenitors or species closely related to them. Fluorescent *in situ* hybridisation showed that *N. paniculata* has one 5S and two 18-5.8-26S rDNA loci whereas *N. undulata* has an additional 18-5.8-26S rDNA locus. *N. rustica* has the sum of the loci found in these putative parents. The sizes of the 18-5.8-26S rDNA loci indicate that the number of rDNA units on the U-genome chromosomes has amplified; perhaps this is associated with a concomitant reduction in the number of units on P-genome chromosomes. Restriction fragment length polymorphism analysis of the intergenic spacer (IGS) of the 18-5.8-26S rDNA units in *N. rustica* and the two progenitor diploids revealed that about 80% of IGS sequences in *N. rustica* are of an *N. undulata* type and 20% of *N. paniculata* type. These data indicate that interlocus sequence homogenisation has caused the replacement of many *N. paniculata*-type IGSs in *N. rustica* with an *N. undulata*-type of sequence. It is probable that subsequent to this replacement there has been sequence divergence at the 5′ end of the IGS. As in tobacco, an allotetraploid between *N. sylvestris* and *N. tomentosiformis*, the direction of the IGS interlocus conversion is towards the paternal genome donor.
Heredity (2003) **91,** 268–275. doi:10.1038/sj.hdy.6800333

Keywords: allopolyploid; rDNA; gene conversion; interphase; concerted evolution

Introduction

Polyploidy is a major force in plant evolution, with as many as 80% of angiosperm species being polyploid (Leitch and Bennett, 1997). Furthermore, DNA sequencing programmes are revealing that many apparently diploid species are palaeopolyploid (Soltis and Soltis, 1999). Plant evolution appears to have involved a series of genome duplication events followed by selection for genome diploidisation. Many genetic events are associated with polyploidy, including chromosomal translocations, gene conversion, as well as loss, gain, amplification and reduction of sequences. However, the universality of these genetic changes, the factors that govern their extent, direction and occurrence are not well understood. How the events attributed to polyploidy differ from events that occur during diploid speciation is the subject of controversy.

One of the best characterised genetic events associated with allopolyploidy is gene conversion at the 18-5.8-26S nuclear ribosomal DNA (rDNA) loci in *Nicotiana tabacum* L. (Kovarik *et al*, 1996; Volkov *et al*, 1999; Lim *et al*, 2000a). *N. tabacum* (tobacco) is an allotetraploid ($2n=4x=48$) that formed within the last 6 million years (Olmstead and Palmer, 1991) between *N. sylvestris* Speg. & Comes ($2n=2x=24$), the maternal S genome donor, and *N. tomentosiformis* L. ($2n=2x=24$), the paternal T genome donor (Goodspeed, 1954; Chase *et al*, 2002; Murad *et al*, 2002). *N. sylvestris* has three rDNA loci, whereas *N. tomentosiformis* has one locus; the sum of these is found in tobacco (Parokonny and Kenton, 1995; Lim *et al*, 2000a). However, an analysis of the rDNA units shows that over 90% of the units are of an *N. tomentosiformis* type whereas the remainder are of an *N. sylvestris* type (Lim *et al*, 2000b). Thus, these data indicate that gene conversion has homogenised most, but not all units.

In this paper we investigate evolutionary processes that have influenced the structure of the 18-5.8-26S rDNA loci in *N. rustica* L. ($2n=4x=48$), a putative allotetraploid between progenitors of the diploid species *N. paniculata* L. ($2n=2x=24$), and *N. undulata* Ruiz and Pavlon ($2n=4x=48$). Phylogenetic studies of the chloroplast sequence suggest that the ancestor of *N. paniculata* and/or *N. knightiana* (section Paniculatae) may be the maternal genome donor of *N. rustic*a (Aoki and Ito, 2000; Chase *et al*, 2003). The closest relative of *N. undulata* (section Undulatae) is *N. glutinosa* although Goodspeed (1954) placed *N. glutinosa* in section Tomentosae. It was Goodspeed's (1954) analyses of morphological data, breeding experiments and karyotypes that led him to the view that *N. paniculata* and *N. undulata* were most closely related to the diploid parents of *N. rustica*, and for this reason these species were analysed here. We used

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Received 24 July 2002; revised 26 February 2003; accepted 14 April 2003

genomic *in situ* hybridisation (GISH) to verify that *N. rustica* is indeed an allopolyploid of these species or a close relative of them. We compared patterns of rDNA evolution in *N. rustica* with *N. tabacum*. We investigated whether the number and location of the rDNA loci is the sum of that found in the putative parents, and whether concerted evolution has altered or homogenised units as has occurred in *N. tabacum*. The results are also interpreted in the light of other rDNA unit arrays previously described in allopolyploids in *Triticum* (Flavell, 1990), *Brassica* (Chen and Pikaard, 1997) and *Gossypium* (Wendel *et al*, 1995a).

Materials and methods

Plant material and chromosome preparations

Root tips of *N. paniculata* L, *N. rustica* L. and *N. undulata* Ruiz & Pavlon (all from USDA, Agricultural Research Station, North Carolina State University, USA) were used in this study. For fluorescent *in situ* hybridisation (FISH), root tips from pot-grown plants were analysed without pretreatment, or following pretreatment with a saturated aqueous solution of Gammexane® (hexachlorocyclohexane, Sigma Aldrich) for 4 h. All root tips were fixed in 3:1 absolute ethanol:glacial acetic acid for more than a week. Chromosome squashes were prepared following enzyme softening of material as described by Leitch *et al* (2001).

Restriction endonuclease digestion and Southern hybridisation

Genomic DNA was extracted from young leaves of these plants using the method of Saghai-Maroof *et al* (1984) with modifications according to Kovarik *et al* (1996). Methods followed those by Sambrook *et al* (1989). DNAs was digested to completion with excess of restriction endonucleases and fractionated in 1% agarose by gel electrophoresis. DNA was transferred to a Hybond N+ membrane (Amersham Pharamacia Biotech.). Southern hybridisation was carried out under high-stringency conditions (Fulnecek *et al*, 2002) using a heat-denatured ^{32}P-labelled rDNA probe that was either (i) a 1.7 kb *Eco*RI fragment of the 18S rRNA gene subunit from *Solanum lycopersicum* L. (tomato, Kiss *et al*, 1989b, accession number X51576) or (ii) a 220 bp fragment of the 26S rRNA gene subunit from *S. lycopersicum* (Kiss *et al*, 1989a, accession number X13557) obtained by PCR amplification of the region between +2901 (5′-GAA TTC ACC CAA GTG TTG GGA T-3′) and +3121 (5′-AGA GGC GTT CAG TCA TAA TC-3′) with respect to the start of the 26S rDNA subunit (Kiss *et al*, 1989a). Labelling of DNA probes was carried out by a random primed method using ^{32}P-dCTP (Dekaprime kit, Ambion™, USA).

Probes for *in situ* hybridisation

(1) *N. undulata* total genomic DNA was labelled by nick translation with digoxigenin-11-dUTP (Roche Biochemicals) and *N. paniculata* total genomic DNA with biotin-16-dUTP (Sigma Aldrich) following Leitch *et al* (2001).
(2) The entire plasmid containing pTa71 (isolated by Gerlach and Bedbrook, 1979), which includes the 18-5.8-26S rDNA subunits and the intergenic spacer (IGS) isolated from *Triticum aestivum*, was labelled with digoxigenin-11-dUTP or biotin-16-dUTP by nick translation.
(3) pTZ19-R: the 5S rDNA unit isolated from *N. rustica* (Venkateswarlu *et al*, 1991) was labelled with digoxigenin-11-dUTP or biotin-16-dUTP by nick translation.

Fluorescent ***in situ*** hybridisation (FISH): FISH was carried out as described by Leitch *et al* (1994), with modifications as described by Lim *et al* (1998). Briefly, slides were pretreated with 100 μg/ml RNase A for 1 h and 0.25 μg/ml pepsin for 5 min followed by denaturation in 70% formamide in 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate) at 70°C for 2 min. The hybridisation mix contained 50% (v/v) formamide, 10% (w/v) dextran sulphate and 0.1% (w/v) sodium dodecyl sulphate in 2 × SSC. For GISH, the hybridisation mixture contained 8 μg/ml digoxigenin-labelled *N. undulata* DNA and 8 μg/ml biotin-labelled *N. paniculata* DNA. A concentration of 4 μg/ml DNA was used in the mixture when using the cloned probes pTa71 and pTZ19-R. After overnight hybridisation at 37°C, the slides were washed in 20% (v/v) formamide in 0.1 × SSC at 42°C at an estimated hybridisation stringency of 80–85%. Sites of probe hybridisation were detected using 20 μg/ml fluorescein conjugated antidigoxigenin IgG (Roche Biochemicals) and 5 μg/ml Cy3 conjugated avidin (Amersham Biosciences). Chromosomes were counterstained with 2 μg/ml DAPI (4′,6-diamidino-2-phenylindole) in 4 × SSC, mounted in Vectashield medium (Vector Laboratories), examined using a Leica DMRA2 epifluorescent microscope and photographed with an Orca ER camera. Images were processed for colour balance, contrast and brightness uniformly.

Results

In situ hybridisation showing the distribution of ribosomal DNA

Using FISH, it was shown that *N. paniculata* has two loci (four sites) of 18-5.8-26S rDNA at the distal end of the short arm of two metacentric chromosomes (Figure 1a). One of these loci was larger than the other, enabling the homologues to be distinguished at metaphase (Figure 1a) and interphase (Figure 2a). Double labelling with 5S rDNA reveals a single locus near the centromere of an additional metacentric chromosome (Figure 1a). In contrast, FISH to *N. undulata* reveals three loci of 18-5.8-26S rDNA at the distal end of the short arm of three metacentric chromosomes, and one of these chromosomes carries a more proximal 5S rDNA locus (Figure 1b). *N. rustica* has the sum of the loci observed in these putative parents (Figure 1c).

GISH can be used to distinguish the genomic origin of the chromosomes in allopolyploids (Heslop-Harrison, 1992; Gill and Friebe, 1998) and has been used to confirm the ancestry of allopolyploids in a number of species including tobacco (Parokonny and Kenton, 1995; Lim *et al*., 2000a). Figure 1d shows that the method distinguishes between chromosomes inherited from progenitors of *N. undulata* and *N. paniculata*, the U- and P-genomes of *N. rustica* respectively. Even though the positions and numbers of the rDNA loci in *N. rustica*

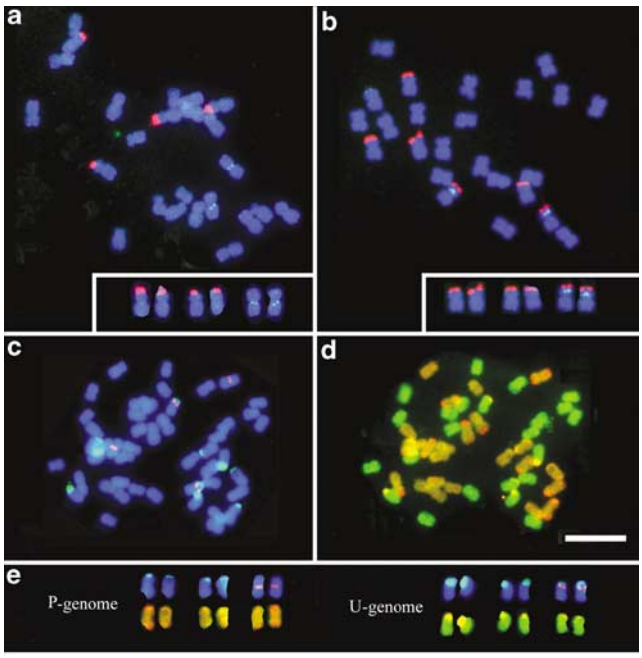

Figure 1 FISH to metaphase spreads of *N. paniculata* (**a**) and *N. undulata* (**b**) probed with biotin-labelled pTa 71 (pink) and digoxigenin-labelled pTZ19-R (green) to reveal sites of 18-5.8-26S rDNA and 5S rDNA loci, respectively. (**c**) Metaphase of *N. rustica* probed with digoxigenin-labelled pTa 71 and biotin-labelled pTZ19-R. The counterstain is DAPI (blue) for DNA. In (**d**), the metaphase shown in (**c**) was reprobed by GISH using total genomic DNA from *N. undulata* (green) and *N. paniculata* (orange). This enables the genomic origin of the chromosomes to be determined (**e**). Those chromosomes in (**c**) and (**d**) that carry rDNA have been given a genome identity. Scale bar 10 μm.

occur as the sum of the organisation found in the parents (Figure 1a and b), there are some notable differences. The two largest 18-5.8-26S rDNA loci in *N. rustica* occur on U-genome chromosomes and can be distinguished from each other by size (Figure 1c–e), and the P-genome 18-5.8-26S rDNA loci are small compared with *N. paniculata*. Assuming that the rDNA signal distribution reflects that found in the true parents of *N. rustica*, the data indicate copy number expansion at one or two U-genome loci, perhaps with a concomitant reduction at P-genome loci.

Many hundreds of meristematic, circular interphase nuclei were examined from several gammexane-treated root tips of the three species analysed. The two 18-5.8-26S rDNA loci in *N. paniculata* are typically expressed at interphase, with the proximal end of the locus being decondensed and the distal end remaining condensed and heterochromatic (Figure 2a and b). Likewise in *N. undulata*, the three 18-5.8-26S rDNA loci are decondensed in a similar manner (Figure 2c and d). In interphases of *N. rustica*, up to four (often two) of the 10 sites are condensed and unassociated with nucleoli (two sites like this are indicated in Figure 2e). The two largest 18-5.8-26S rDNA loci in *N. rustica*, carried on U-genome chromosomes, show interlocus variability in the level of chromatin decondensation, but with most condensed rDNA occurring at the distal end of the locus (Figure 2e and f). The third rDNA locus on a U-genome chromosome is not materially larger than the P-genome loci, and the sites are not readily distinguishable at interphase. But at metaphase, the third U-genome chromosome carrying 18-5.8-26S rDNA can be identified by the linked 5S rDNA locus. In these cells this U-genome chromosome can be seen to carry a secondary constriction suggesting locus activity at the preceding interphase (Figure 2g and h, arrow). An examination of *N. rustica* root-tip cells without pretreatment shows that the decondensed rDNA is more dispersed, but the profile of locus decondensation remained similar with silent sites unassociated with nucleoli apparent at interphase (Figure 2 i–l). Thus, the rDNA loci of *N. rustica* show variable activity both between and within loci as reported for other plant species (Vieira *et al*, 1990) and that the least active sites appear to occur on P-genome chromosomes.

Genetic structure of the IGS in *N. rustica* and its putative parents

The genetic structure of the IGS of the 18-5.8-26S rDNA gene unit in *N. rustica* and its putative diploid parents *N. paniculata* and *N. undulata* was analysed using Southern hybridisation. Figure 3a shows *Hin*d III-digested DNA of *N. paniculata*, *N. rustica* and *N. undulata* probed with the 26S rDNA probe. The restriction profile shows a band of 9.5 kb in *N. paniculata* and slightly greater than 10 kb in *N. undulata*. The same bands are revealed with the 18S rDNA probe (data not shown), providing evidence that these restriction fragments are monomeric units of the 18-5.8-26S rDNA transcribed genic region and the IGS. A similar-sized 18-5.8-26S rDNA unit is found in related *Nicotiana* species (Kovarik *et al*, 1996; Volkov *et al*, 2001). However, *N. rustica* does not have the sum of the bands seen in *N. paniculata* and *N. undulata*. Instead a more complex profile is observed with several bands occurring between 9.5 and 10.5 kb, and only some of these are directly coincident with those seen in the diploids.

A range of restriction enzymes was used to analyse size heterogeneity in the 18-5.8-26S rDNA unit of *N. rustica* and to investigate any polymorphisms in the IGS of all three species. When there are two or more restriction sites in the rDNA unit, and one or more are in the IGS, then the 18S rDNA probe identifies polymorphisms at the 3′ end of the IGS. Digestion with *Ssp*I and *Dra*I restriction enzymes and probing with 18S rDNA generates fragments that show little polymorphism between species (Figure 3b). In contrast when genomic DNA is restricted with *Eco*RV a distinct species-specific hybridisation profile is observed, allowing the two diploid species to be distinguished. With *Eco*RV it is evident that only a small fraction (ca 20%) of the rDNA restriction fragments in *N. rustica* appear to be similar to those in *N. paniculata* (Figure 3b arrow) — (P-type units in *N. rustica*), — whereas the majority of IGS copies have a restriction profile that closely resembles *N. undulata*, U-type units.

Reprobing the membrane containing *Ssp*1, *Dra*I and *Eco*RV digested DNA (Figure 3b) with the 26S rDNA probe (Figure 3c) reveals IGS polymorphism at the 5′ end of the sequence. In *Eco*RV digested DNA, a small fraction of the bands in *N. rustica* are shared with *N. paniculata* and *N. undulata*, whereas in *Ssp*I and *Dra*I restricted DNA only some bands are shared with *N. undulata*. Most of the hybridisation bands in *N. rustica* are unique, indicating considerable genetic change at the 5′ end of

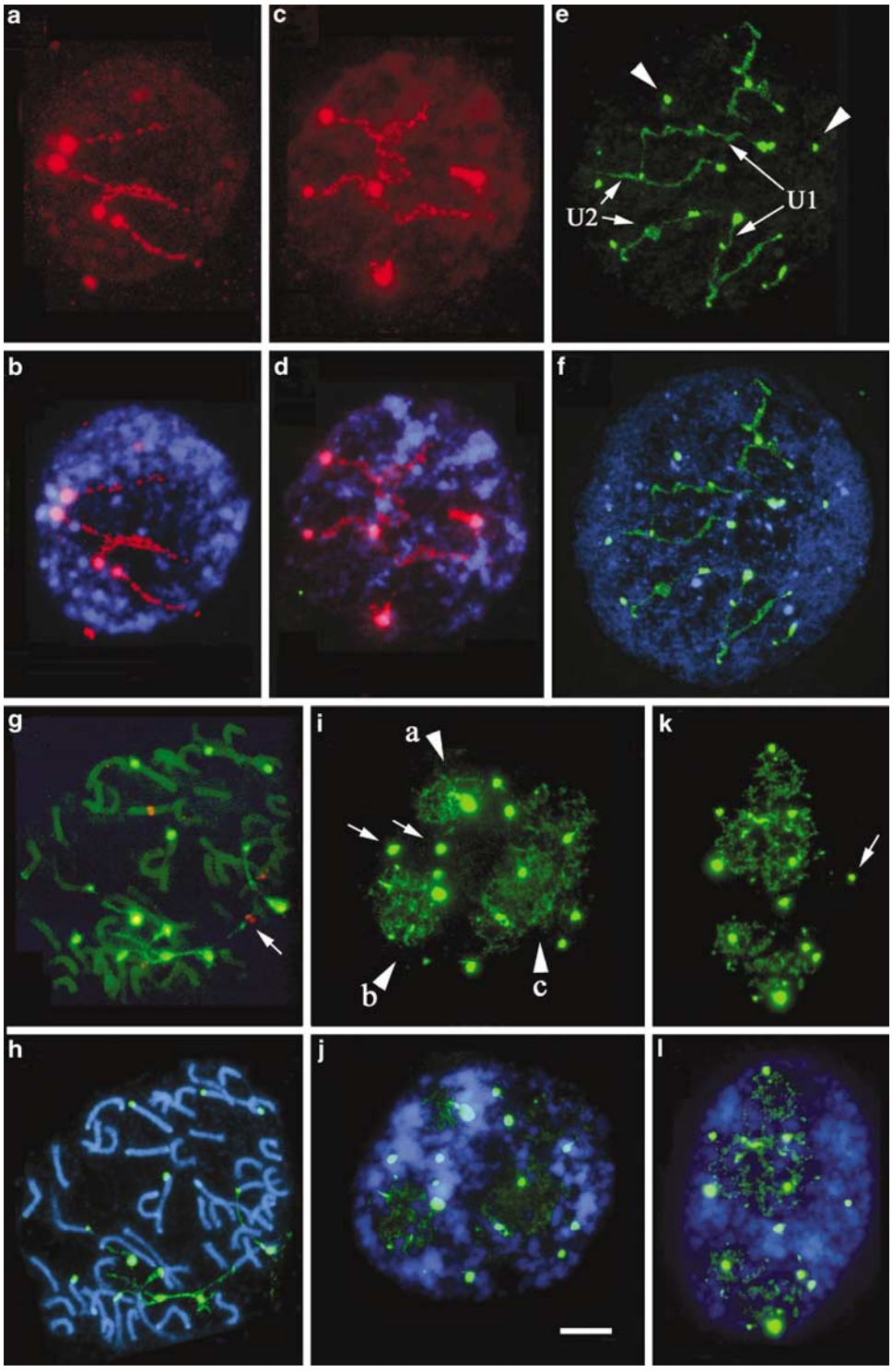

Figure 2 Interphases of *N. paniculata* (**a,b**), *N. undulata* (**c,d**) and *N. rustica* (**e,f**) probed with pTa71 labelled either with digoxigenin-FITC (yellow/green) or biotin–avidin Cy3 (orange/red). The counterstain for DNA is DAPI (blue). (**a–h**). Cells from gammexane-treated roots. (**a**) Two pairs of different-sized 18-5.8-26S rDNA loci and differential decondensation withinin each locus are shown. The red dot outside the nucleus is background. (**c,d**) The three pairs of rDNA loci show signs of decondensation at interphase. The arrow heads in (**e**) show small rDNA sites that are condensed, inactive and unassociated with nucleoli. The arrows point to the two largest rDNA loci from U-genome chromosomes. Each of these sites show variable condensation. (**g**) Pro-metaphase of *N. rustica* probed with digoxigenin-labelled pTa71 (18-5.8-26S rDNA, green) and biotin-labelled pTZ19-R (5S rDNA, red) rDNA. The arrow points to a U-genome chromosome carrying both 5S and 18-5.8-26S rDNA loci, the latter having a secondary constriction. (**i–l**) Interphases from untreated root tips (without gammexane). The nucleus (**i,j**) has three nucleoli (a–c) containing dispersed, probably active rDNA and peripheral blocks of condensed rDNA. There are at least one (**k,l**) or two (**i,j**) rDNA sites (arrows) that are condensed, heterochromatic and unassociated with nucleoli. The scale bar represents 13 μm (**a–d**), 10 μm (**e–h**), and 12 μm (**e–h**).

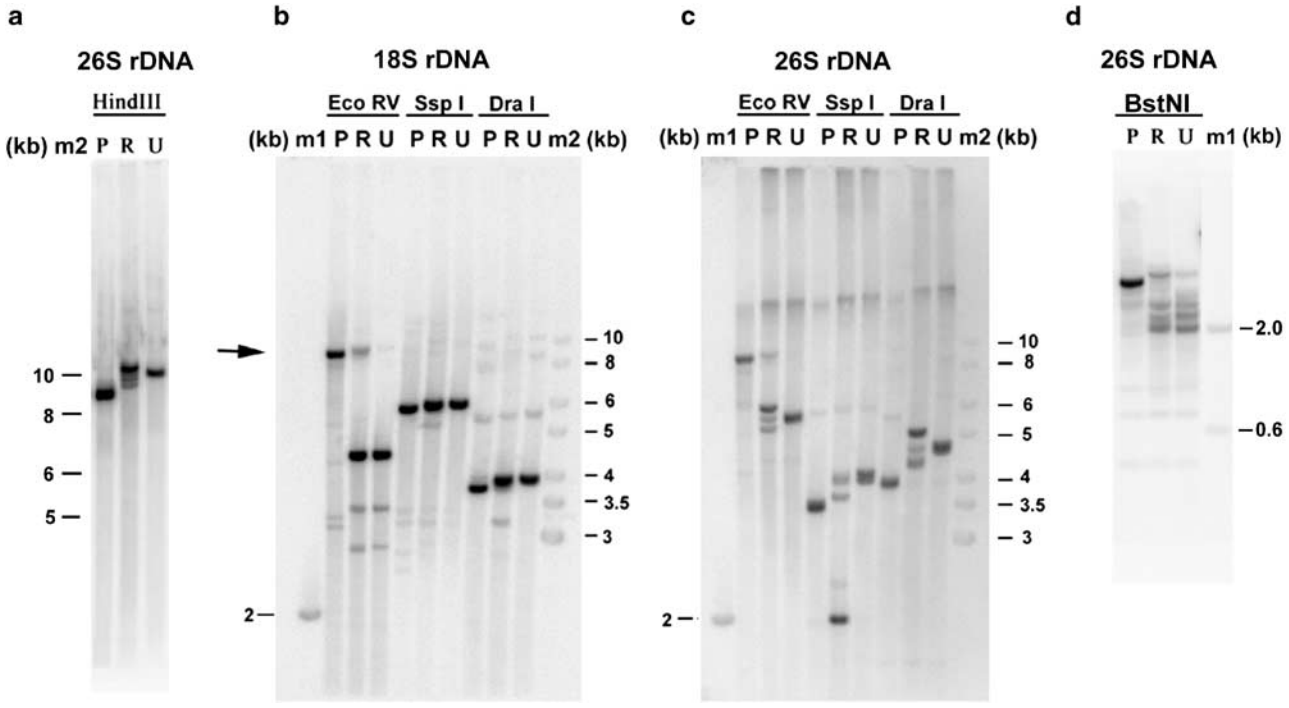

Figure 3 Southern hybridisation showing the overall structure of 18-5.8-26S rDNA units in *N. paniculata* (P), *N. rustica* (R) and *N. undulata* (U). The genomic DNA was cut with restriction enzymes and probed with 26S (**a**, **c**, **d**) and 18S (**b**) rDNA as indicated. The arrow in **b** indicates those units in *N. rustica* that are similar to units found in *N. paniculata*. m1: 100 bp DNA ladder (Gibco BRL); m2: 1 kb ladder (MBI Fermentas).

the IGS (towards the 26S rRNA gene) in the vast majority of *N. rustica* units. Nevertheless, an *N. undulata*-like character of *N. rustica* units can be observed in BstN1 restricted DNA probed with 26S rDNA (Figure 3d). Reprobing the *Bst*NI-digested genomic DNA with the 5S rDNA probe revealed that *N. rustica* has a pattern that is additive of that seen in the parents (data not shown).

Methylation patterns in *N. rustica* and its putative parents

Digestion with *Eco*RV and probing with 18S or 26S rDNA probes produced species-specific restriction patterns that can be exploited to determine levels of DNA methylation in parental rDNA gene families in *N. rustica*. Double digests of genomic DNAs were carried out using *Eco*RV and the cytosine methylation-sensitive enzymes *Fnu*4HI, *Xho*I and *Pst*I (Figure 4). *Xho*I is sensitive to methylation at CTmCGAG and has a conserved target site within the 26S gene of most plant species (as determined from plant DNA sequence databases). When *N. rustica* is digested with methylation-insensitive *Eco*RV, a small proportion of units appear like *N. paniculata* as shown previously (Figure 3b). These units migrating as an ~9 kb *Eco*RV band are unaffected by Xho I digestion (Figure 4) in *N. rustica*, indicating that they are heavily methylated. However, in *N. paniculata* a significant fraction of this 9 kb *Eco*RV band is digested by *Xho*I into 5.5 kb (strong) and 7.4 kb (weak) bands. It is this undermethylated fraction of rDNA units that is missing in *N. rustica*. Using *Fnu*4HI (sensitive to methylation at GmCNGC) in the double digest with *Eco*RV, and probing with 18S rDNA generates a complex array of bands (Figure 4). Once again many of the bands in *N. rustica* are similar to bands in *N. undulata* and absent in *N. paniculata*. The enzymes *Xho*I, *Pst*I (sensitive to methylation at mCTGmCAG) and

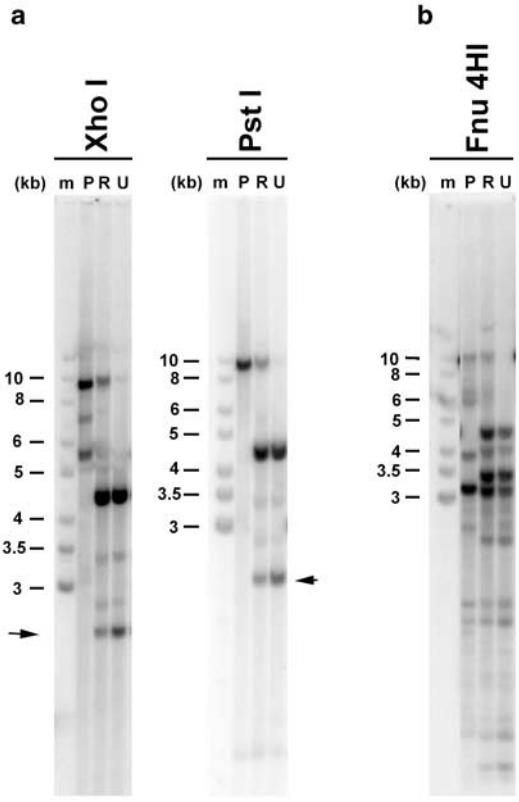

Figure 4 Southern hybridisation to determine methylation levels of 18-5.8-26S rDNA units in *N. paniculata* (P), *N. rustica* (R) and *N. undulata* (U). The genomic DNA was digested with *Eco*RV and subsequently with methylation-sensitive enzymes as indicated. The blots were probed with 18S rDNA. The arrow shows short bands that are more prominent relative to the longer bands in *N. undulata* than is observed in *N. rustica*. m: 1 kb DNA ladder.

*Fnu*4HI also reveal subtle differences in methylation levels between *N. undulata* and *N. rustica*, with a larger fraction of smaller fragment sizes in *N. undulata*, indicating slightly less cytosine methylation compared with *N. rustica*.

Discussion

The numbers and locations of 18-5.8-26S and 5S rDNA loci in *N. rustica* and the strong GISH signal strongly support the hypothesis that *N. rustica* is an allopolyploid between *N. paniculata* and *N. undulata*, their progenitors, or closely related species. It is apparent that since allopolyploidy there has been little change in the overall structure of the chromosomes. Furthermore, if there was substantial intermixing of the genomes subsequent to allopolyploid formation, through, for example, retrotranspostion or sequence homogenisation, it would be unlikely that GISH would work effectively. The fact that it does argues for little intermixing of parental genomes over large tracks of the genome. However, there has been considerable change at the 18-5.8-26S rDNA loci.

In *N. paniculata*, which has two 18-5.8-26S rDNA loci, there is a uniform length of the basic 18-5.8-26S rDNA subunit, and restriction analysis indicates one predominant IGS type (Figures 3 and 5). In *N. undulata*, which has three 18-5.8-26S rDNA loci, there is also a uniform length of the 18-5.8-26S rDNA subunit, and only a few polymorphisms in the IGS are revealed with some enzymes (eg *Bst* NI) indicating several (three or four) similar subunit types (Figures 3d and 5). In contrast, *N. rustica* has at least three classes of rDNA unit length over the five rDNA loci and considerable heterogeneity of IGS at the 5′ end of the sequence (Figures 3 and 5). In the majority (80%) of these units, there is high similarity at the 3′ end of the IGS to sequences in *N. undulata*.

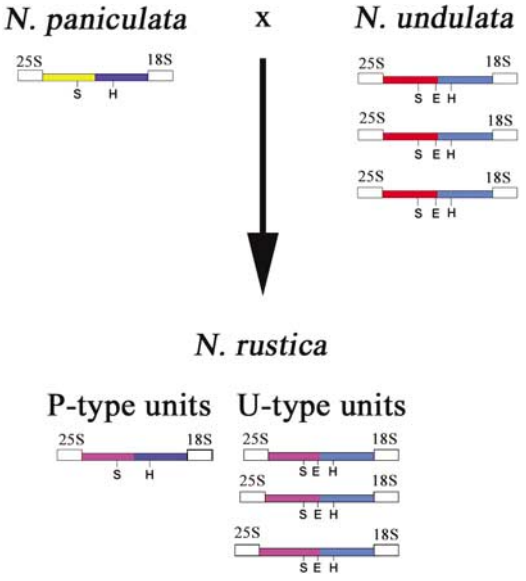

Figure 5 Schematic diagram showing the IGS structure between the 26S and 18S rDNA subunits of *N. paniculata*, *N. undulata* and *N. rustica*. The colour coding gives an indication of similarity of units. The relative positions of *Eco*RV (E), *Hin*dIII (H) and *Ssp*I (S) target sites are shown in rDNA unit variants.

The restriction patterns revealed with the 18S rDNA probe indicate sequence homogenisation across many rDNA units in *N. rustica*. The *N. paniculata*-type subunits are under-represented in *N. rustica*, and this may have arisen via recombination between P- and U-genome units within the IGS followed by homogenisation, so that now only *ca* 20% of the units have an *N. paniculata*-like character. It is possible that this process happened early in the divergence of *N. rustica*, and it may have been associated with allopolyploidy itself, which can stimulate genome reorganisation in some species (Ozkan *et al*, 2001). It is also likely that the length of the IGS slowly converges to a uniform size by dynamic compensation of deletions and amplifications of IGS subrepeats (Volkov *et al*, 1999).

Evidence from plastid DNA sequence data indicates that an ancestor of *N. paniculata* is the maternal genome donor (Aoki and Ito, 2000). Gill (1991) suggested in the nucleocytoplasmic interaction hypothesis that the paternal genome of newly formed allopolyploids was most likely to reorganise to accommodate an alien cytoplasmic background. However in *N. rustica*, the units of rDNA from *N. paniculata*, the putative maternal genome, seem to have undergone the greatest change. Thus, as in tobacco (Volkov *et al*, 1999; Lim *et al*, 2000b), interlocus gene conversion has occurred so that the IGS of the paternal genome donor is predominating. Furthermore, combined GISH and rDNA *in situ* hybridisation revealed that the largest, most active rDNA loci are on the U-genome chromosomes, indicating expansion of the *N. undulata*-type units at these loci. The small size of the P-genome loci might suggest that this is associated with a reduction in the number of units on the P-genome chromosomes.

The character of the IGS in *N. rustica* appears different when 26S rDNA is used as the probe. The probe detects polymorphisms occurring predominantly at the 5′ end of the IGS, and with most enzymes a unique pattern of bands in *N. rustica* was observed. Only one enzyme (*Bst*NI) revealed an *N. undulata*-like pattern similar to those seen with the 18S rDNA probe. These data indicate either IGS sequence divergence subsequent to homogenisation or a different IGS character in the true parent of *N. rustica*. If sequence divergence is influencing the 5′ end of the IGS more than the 3′ end of the sequence, this probably reflects functional constraints associated with the promoter, regulator and externally transcribed spacer sequences found in the 3′ region.

Homogenisation of rDNA is often, but not always, observed at rDNA loci. In wheat (*Triticum aestivum*), a 10 000-year-old allohexapolyploid, subrepeats are found in the IGS (Barker *et al*, 1988) as in tobacco (Volkov *et al*, 1999). In wheat, the structure of the A-type subrepeat varies little, but their copy number differs among loci and cultivars (Flavell, 1990). In wild populations of tetraploid wheats, the IGS in some populations is heterogeneous whereas in others it is homogeneous (Flavell *et al*, 1986), indicating both divergence, homogenisation and fixation of subrepeat number (Flavell, 1989), processes that are probably similar to what has occurred in *N. rustica*. In *Gossypium* there has also been interlocus concerted evolution of rDNA units in several allopolyploid species, and homogenisation has occurred bidirectionally in allopolyploid species with similar genetic backgrounds (Wendel *et al*, 1995b). However

gene conversion appears not to have homogenised loci in *Brassica* allotetraploids (Chen and Pikaard, 1997).

Lim *et al* (2000b) argued that a major controlling factor for the occurrence and extent of sequence homogenisation by gene conversion was rRNA gene activity at interphase when the DNA is most decondensed. It is possible that sequence homogenisation occurs when activity at interphase is coupled with mutual homology between IGSs of different rDNA units. The direction of change may be random, except that the most abundant sequence type would most likely be fixed. If this reasoning is correct and there is high mutual homology between IGS sequences, then those variants associated with active, expressed rDNA units at interphase are likely to predominate. Cytosine methylation of rDNA units was higher in *N. rustica* compared with the diploid progenitor species, particularly at the minor fraction of units that have not been homogenised and retained the *N. paniculata*-like character (see *Xho*I and *Fnu*4HI digests, Figure 4). Similarly, in *N. tabacum* the minority *N. sylvestris*-type units are heavily methylated (Lim *et al*, 2000b). Heavily methylated units are not usually active at interphase; indeed inactive rDNA loci can be experimentally activated by the addition of demethylating agents (Vieira *et al*, 1990; Chen and Pikaard, 1997). If Lim *et al* (2000b) are correct, then it can be predicted that there is lower activity of the rDNA loci on the P-genome chromosomes of *N. rustica* and this is a factor contributing to the maintenance of some *N. paniculata*-like units in the allotetraploid. While there is some evidence of reduced 18-5.8-26S rDNA activity in the P-genome chromosomes of root-tip interphases, more direct proof that this is inhibiting interlocus homogenisation requires the analysis of rDNA condensation in undistorted nuclei, from germ-line tissue of resynthesised synthetic *N. rustica*, with the parental origin of the rDNA units and loci identified by specific *in situ* markers.

Acknowledgements

We thank NERC and the Grant Agency of the Czech Republic (Grants 204/01/0313 and 521/01/0037) for funding. We thank Miss D Saikia and Mr J Clarkson for assistance, and Professor M Chase and Dr I Leitch for helpful comments.

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