Chromosome Res (2013) 21:375–381
DOI 10.1007/s10577-013-9363-y

Tobacco karyotyping by accurate centromere identification and novel repetitive DNA localization

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Received: 6 February 2013 / Revised: 18 April 2013 / Accepted: 30 April 2013 / Published online: 23 May 2013

Abstract Tobacco (*Nicotiana tabacum*) is an amphidiploid species ($2n=4x=48$, genome constitution SSTT) derived from a natural hybrid between *Nicotiana sylvestris* ($2n=2x=24$, SS) and *Nicotiana tomentosiformis* ($2n=2x=24$, TT). Genomic in situ hybridization (GISH), using the genomic DNA from these ancestral species as probes, revealed the chromosomal origins (S or T) and the occurrence of intergenomic translocations in *N. tabacum*. Fluorescence in situ hybridization (FISH) was also used to distinguish between chromosomes. However, the use of repetitive DNA sequences as probes for FISH analysis is limited by an inability to identify all chromosomes. In addition to this limitation, the occurrence of chromosomal tertiary constrictions can easily lead to the misclassification of chromosomes. To overcome these issues, immunostaining with anti-*N. tabacum* centromere-specific histone H3 antibody was carried out to determine the centromere position of each chromosome, followed by FISH analysis with ten distinct repetitive DNA probes. This approach allowed us to identify 22 of the 24 chromosome pairs in *N. tabacum* and revealed novel intergenomic chromosome rearrangements and B-chromosome-like minichromosomes. Hence, the combination of immunostaining with FISH and GISH is critical to accurately karyotype tobacco.

Keywords *Nicotiana tabacum* · fluorescence in situ hybridization (FISH) · genome in situ hybridization (GISH) · immunostaining · centromere-specific histone H3 (CENH3)

Abbreviations

CENH3	Centromere-specific histone H3
DAPI	4,6-diamino-2-phenylindole
DIG	Digoxigenin
FISH	Fluorescence in situ hybridization
GISH	Genomic in situ hybridization
NtCENH3	*Nicotiana tabacum* centromere-specific histone H3
PBS	Phosphate-buffered saline

Introduction

Among Solanaceae, *Nicotiana* is a relatively large genus containing 76 species, approximately half of which are amphidiploids (Knapp et al. 2004). *Nicotiana tabacum* originated from a natural amphidiploid in America, and a number of cultivars have since been developed and

Responsible Editor: Hans de Jong.

Electronic supplementary material The online version of this article (doi:10.1007/s10577-013-9363-y) contains supplementary material, which is available to authorized users.

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grown widely throughout the world. Thus, chromosomes of *N. tabacum* (2*n*=4*x*=48) can be divided into two components: one from *Nicotiana sylvestris* (2*n*=2*x*=24, S genome), and the other from *Nicotiana tomentosiformis* (2*n*=2*x*=24, T genome) (Fulnecek et al. 2002; Goodspeed 1954; Kitamura et al. 2005; Lim et al. 2000). Although *N. tabacum* chromosomes are relatively small, its karyotype was produced by Goodspeed (1954) more than a half century ago. According to his data, *N. tabacum* consists of ten median, five submedian, and nine subterminal chromosomes (10m+5sm+9st), whereas one of the putative ancestors, *N. tomentosiformis* (or *N. otophora*), consists of 7m+1st, and the other, *N. sylvestris*, consists of 3m+5sm+4st. These results supported a model whereby simple amphidiploid formation occurred between the two ancestor species, followed by amphidiploid stability. However, a molecular and cytogenetic technique, genomic in situ hybridization (GISH), has revealed that up to nine intergenomic chromosomal rearrangements have occurred in the natural genotypes of *N. tabacum* (Kenton et al. 1993). Despite the relatively frequent occurrence of chromosomal rearrangements in this species, such chromosomal rearrangements were not found in new synthetic allotetraploids (Lim et al. 2006).

In combination with GISH, fluorescence in situ hybridization (FISH), using repetitive DNA sequences as probes, has been used to identify chromosomes (Lim et al. 2000; Lim et al. 2004; Moscone et al. 1996). For example, Leitch and his colleague hybridized six distinct DNA probes to *N. tabacum* cv. 095-55 (2*n*=2*x*=48), but only 18 pairs of chromosomes displayed FISH signals, while the remaining six pairs showed none (Lim et al. 2000). Because there are limited numbers of DNA markers for FISH analysis in tobacco, comparative karyotype analyses among the cultivars have not been thoroughly conducted. In addition, determining the accurate centromere positions for some chromosomes is difficult because of the occurrence of tertiary constrictions (Goodspeed 1954). Therefore, in this study, immunostaining using an antibody against tobacco centromere-specific histone H3 was first carried out to determine the centromere positions, followed by FISH analyses using ten distinct repetitive DNA sequences as probes. With this approach, we identified 22 of the 24 chromosome pairs of *N. tabacum* and novel aberrant chromosomes including a supernumerary B-chromosome-like minichromosome.

Materials and methods

Plant material

A cultivar of allotetraploid tobacco (*N. tabacum* L. cv. Petit Havana SR1, 2*n*=4*x*=48) was used in this study. The seeds, which were a gift from Japan Tobacco Inc., were germinated in soil and plants were grown in a greenhouse under natural condition.

Fluorescence in situ hybridization

Chromosome spreads for FISH experiments were prepared as described previously (Shibata and Hizume 2002). Roots of plants were treated with 0.05 % (*w*/*v*) colchicine for 1.5 h at room temperature and fixed in acetic acid: ethanol (1:3). The fixed root tips were treated with an enzyme mixture (2 % (*w*/*v*) Cellulase Onozuka RS (Yakult, Japan) and 0.5 % (*w*/*v*) Pectolyase Y-23 (Kyowa Hakko, Japan) in citrate buffer at pH 5.5) for 30 min at 37 °C. The macerated root tips were squashed on glass slides with cover slips, and the cover slips were later removed on dry ice. FISH was performed as described previously (Murata et al. 2008). Probes used in this study are listed in Table 1. DNA probes were labeled by nick translation using a DIG-Nick Translation Mix (Roche Diagnostics, Japan) or a Biotin-Nick Translation Mix (Roche Diagnostics). The DIG- and biotin-labeled probes were visualized using rhodamine-conjugated anti-digoxigenin antibody (Roche Diagnostics) and Alexa Fluor 488-conjugated streptavidin (Life Technologies, Japan), respectively. Chromosomes were counterstained with 0.1 μg/ml 4,6-diamino-2-phenylindole (DAPI). FISH signals and stained chromosomes were captured using a chilled charge-coupled device camera (Axiocam HR, Carl Zeiss, Germany) and images were pseudo-colored and processed using Axiovision software (Carl Zeiss). For multi-color FISH, cover slips were removed from the slides after imaging, and the slides were then fixed in acetic acid/ethanol (1:3) for 20 min, air-dried, and treated with different FISH probes; this procedure was repeated six times for each slide.

Immunostaining

Roots were fixed with 4 % (*w*/*v*) paraformaldehyde, 0.1 % (*v*/*v*) Tween 20 in phosphate-buffered saline

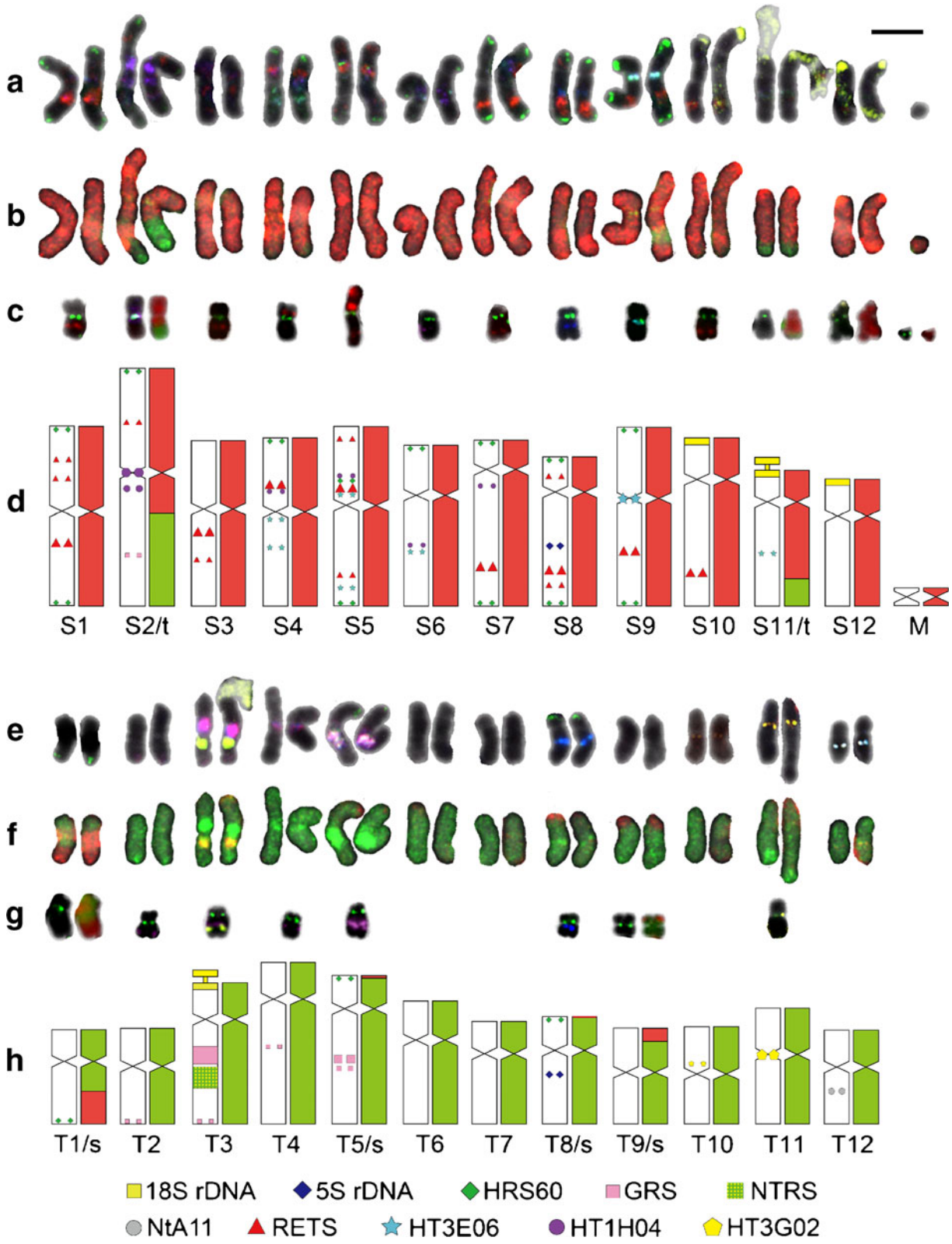
(PBS) for 30 min at room temperature. The fixed root tips were washed with PBS and treated with the enzyme mixture (2 % (*w*/*v*) Cellulase Onozuka RS (Yakult) and 0.5 % (*w*/*v*) Pectolyase Y-23 (Kyowa Hakko) in citrate buffer at pH 5.5) for 30 min at 37 °C. The macerated root tips were washed with PBS and squashed on glass slides with cover slips. The cover slips were later removed on dry ice. To detect centromere-specific histone H3 in *N. tabacum* (NtCENH3), anti-NtCENH3 antibody (Nagaki et al. 2009), diluted 1:100 with PBS, was applied to the slides. After washing in PBS, the antibody was visualized with a 1:100 dilution of anti-rabbit Alexa Fluor 555 antibody (Life Technologies). Chromosomes were counterstained with 0.1 μg/ml DAPI, and the chromosomes and signals were detected as described in the FISH analysis. After capturing images, cover slips were removed from the slides. The slides were then fixed in acetic acid: ethanol (1:3) for 20 min and air-dried. The dried slides were later used for FISH, using repetitive DNA sequences or genomic DNA as probes.

Results and discussion

Since the pioneering work of Goodspeed (1954), karyotype analyses of *N. tabacum* have been performed by several research groups using FISH in combination with GISH (Kenton et al. 1993; Lim et al. 2000; Lim et al. 2004; Moscone et al. 1996). These groups used five distinct repetitive DNA sequences as FISH probes (18S and 5S rDNAs, HRS60, GRS, and NTRS) (Table 1). We utilized the same repetitive DNA sequences in this study to compare our results with their karyotypes and obtained similar FISH patterns except for HRS60. The HRS60 sequences were mapped to both termini of most S-genome chromosomes (S1/t, S3, S4, S5, S7, S8, and S10) and to the T-genome chromosomes with a translocation (T1/s, T5/s, T7/s and T9/s) in *N. tabacum* cv. 095–55, indicating S-genome specificity and origin (Lim et al. 2000). In the present study with a Petit Havana SR1 cultivar, however, the sequences were detected on eight pairs of S-genome chromosomes (S1, S2/t, S4,

Table 1 FISH and GISH probes used in this study

Probe DNA	Preparation	Reference
18S rDNA	PCR amplified from genomic DNA of *Arabidopsis thaliana* with primers AT18SF (AATGATCCTTCCGCAGGTT) and AT18SR (TACCTGGTTGATCCTGCCAGT)	Shibata and Murata (2004)
5S rDNA	PCR amplified from genomic DNA of *Nicotiana tabacum* with primers 5SL1 (CGGTGCATTAATGCTGGTAT) and 5SL2 (CCATCAGAACTCCGCAGTTA)	Hizume (1993)
HRS60	PCR amplified from genomic DNA of *N. tabacum* with primers HRS60-F (AAACAATTTGCACCCACTCG) and HRS60-R (TATGCCGTATTTGATGTCCG)	Koukalova et al. (1989) and Lim et al. (2000)
GRS	PCR amplified from genomic DNA of *N. tabacum* with primers GRS-F (CACGAAAATATGGGA ATCGG) and GRS-R (TATAGCCCACGCCTTTCG)	Gazdova et al. (1995)
NTRS	PCR amplified from genomic DNA of *N. tabacum* with primers NTRS-F (GAACCACCATCATCAACACA) and NTRS-R (ATGCCACTTAGGTTTCAAGG)	Matyášek et al. (1997)
HT1H04	Cloned from genomic DNA of *N. tabacum*	Nagaki et al. (2012)
HT3E06	Cloned from genomic DNA of *N. tabacum*	Nagaki et al. (2012)
HT3G02	Cloned from genomic DNA of *N. tabacum*	Nagaki et al. (2012)
NtF08 (RETS)	Cloned from genomic DNA of *N. tabacum* cv. Petit Havana SR1	Nagaki et al. (2012) and Suzuki et al. (1994)
NtA11	Cloned from genomic DNA of *N. tabacum* (548 bp)	Nagaki et al. (2012)
Genomic DNA of *Nicotiana sylvestris*	Extracted from leaves	–
Genomic DNA of *Nicotiana tomentosiformis*	Extracted from leaves	–


a
b
c
d
S1
S2/t
S3
S4
S5
S6
S7
S8
S9
S10
S11/t
S12
M
e
f
g
h
T1/s
T2
T3
T4
T5/s
T6
T7
T8/s
T9/s
T10
T11
T12
18S rDNA
5S rDNA
HRS60
GRS
NTRS
NtA11
RETS
HT3E06
HT1H04
HT3G02

◀ **Fig. 1** Karyotype of *N. tabacum* cv. Petit Havana SR1. FISH images (**a**, **e**), GISH images (**b**, **f**), immunostaining images with FISH or GISH (**c**, **g**), and their summarized ideograms (**d**, **h**). Chromosomes in (**a**), (**b**), (**e**), and (**f**) are from the same metaphase plate. Chromosomes in (**c**) and (**g**), which were cut out from different metaphase plates, are labeled by immunostaining with anti-NtCENH3 antibody (*green*) and by FISH or GISH (images on the right show S2/t, S11/t, T1/s, and T9/s). The colors of FISH signals on chromosomes (**a**, **b**, **e**, and **f**) correspond to those of the FISH probes shown at the *bottom*. *Red* and *green* GISH signals in (**b**) and (**f**) are derived from the genomic DNA of *N. sylvestris* and *N. tomentosiformis*, respectively. *Scale bar*, 10 μm

S5, S6, S7, S8, and S9) (Fig. 1a, d) and three pairs of T-genome chromosomes with translocations (T1/s, T5/s and T8/s), all of which have the S-genome segments at either or both termini (Fig. 1e, h). This discrepancy is possibly caused by the difference between the cultivars used. A combination of GISH and FISH with five repetitive DNA probes (18S rDNA, 5S rDNA, HRS60, GRS, and NTRS) identified thirteen pairs of chromosomes in this study with the cultivar, Petit Havana SR1 (S2/t, S5, S8, S10, S11/t, S12, T1/s, T2, T3, T4, T5/s, T8/s, and T9/s).

To identify more chromosome components of *N. tabacum*, five repetitive DNA FISH probes from our previous study (Nagaki et al. 2012) were added to the present study (Table 1). In addition, immunostaining with an anti-NtCENH3 antibody (Nagaki et al. 2011) was used to determine accurate centromere positions, prior to FISH analysis, because the centromere positions of several chromosomes were not easily identified due to a multi-constriction-like structure, originally characterized by Goodspeed (1954). Chromosomes that showed no detectable FISH signals after immunostaining with anti-NtCENH3 antibody (T6, T7, and T10), were compared to a karyogram, previously reported by Moscone et al. (1996), to determine their centromere positions. Six intergenomic translocations, revealed by GISH in this study (Fig. 1b, d, f, h; Fig. S1 in the Electronic supplementary material (ESM)), were classified as S-genome or T-genome chromosomes based on the genome sequence the centromeric region; the second genome involved in the translocation was denoted by a backslash and lower case, i.e., S2/t, S11/t, T1/s, T5/s, T8/s, and T9/s. The small intergenomic translocations on T5/s and T8/s were first detected in this study (Fig. 1f, h; Fig. S1 in the ESM), and very small chromosomes (M), which displayed S-genome signals by GISH, appeared in some of the cells.

Among the five different DNA sequences utilized as FISH probes, NtF08 was found to have 91 % sequence similarity to repeats containing telomeric stem (RETS) (Suzuki et al. 1994), and therefore, we refer to NtF08 by its original name, RETS (Fig. 1). RETS was isolated from *N. tabacum* cv. Petit Havana SR1 as tandem repeats with a 45-bp repeat unit, consisting of a 35-bp palindromic sequence and a 10- to 11-bp linker sequence (Suzuki et al. 1994). Although the RETS sequences were thought to be rich at chromosome ends,

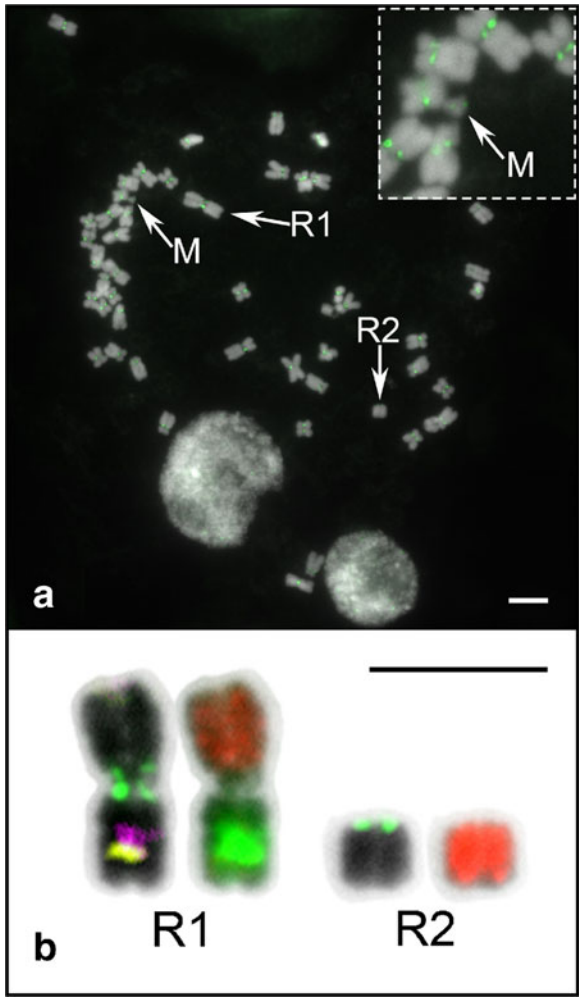


Fig. 2 Unusual chromosomes and the occurrence of micronuclei and chromosome lagging. **a** A mitotic metaphase cell ($2n=48$) immunolabeled with anti-NtCENH3 antibody (*green*). Two unusual chromosomes (*R1* and *R2*) and a minichromosome (*M*) were detected. *Inset* shows the enlarged region containing chromosome M. **b** Magnified images of chromosomes R1 and R2, showing an immunosignal of anti-NtCENH3 antibody (*green*), FISH signals of GRS (*magenta*), and NTRS (*yellow*) and GISH signals from the genomic DNA of *N. sylvestris* (*red*) and *N. tomentosiformis* (*green*). *Scale bar*, 2 μm

our FISH analysis revealed that the sequences are localized preferentially to interstitial regions of the S-genome chromosomes (S1, S2/t, S3, S4, S5, S7, S8, S9, and S10) (Fig. 1c, d). The DNA sequence of NtA11, 548 bp in length, showed 76 % similarity to a sequence registered as microsatellite A30 (GenBank database accession number, DQ865434), which contains AG and/or TG microsatellites; However, no microsatellite sequences were found in NtA11. The DNA sequence localized to a long arm of the T-genome chromosome, T12 that had no prior FISH markers (Fig. 1e, h), thereby making NtA11 a useful FISH probe.

The remaining three clones (HT3E06, HT1H04, and HT3G02), which were isolated as centromere-specific tandem repeats by chromatin affinity purification, were found to be chromosome-specific. HT3E06 shared 80 % sequence similarity to NTS9 (Jakowitsch et al. 1998), the DNA sequence of which was detected as a major pericentromeric signal on chromosome S9 and a minor signal on S11. In contrast to NTS9, which displayed two pairs of FISH signals, the HT3E06 sequence was detected as minor signals on four pairs of the S-genome chromosomes, S4, S5, S6, and S11/t (Fig. 1a, d). HT1H04 produced a major signal on the centromeric region of chromosome S2/t, in addition to a signal on the pericentromeric region of the same chromosome (Fig. 1a, d). Minor signals were also detected on the chromosomes S4, S5 and S6 (Fig. 1a, d). Both HT3E06 and HT1H04 could be good markers for identifying chromosome S6 which had no prior FISH markers. HT3G02 was T-genome-specific, with a strong centromeric signal on chromosome T11, and a minor pericentromeric signal on chromosome T10 (Fig. 1e, h). This result indicates that HT3G02 is a useful probe for identifying chromosomes T10 and T11, which had no prior FISH markers. It should be noted that chromosomes T11 are not heteromorphic, which was caused by folding of the long arm (cf. Nagaki et al. 2012). Although no FISH probes were capable of identifying the remaining chromosomes, T6 and T7, they were distinguishable from each other by their chromosome lengths. The signal patterns obtained in this study are also summarized in Table S1 in the ESM. Consequently, the five repetitive DNA sequences added as FISH probes was found to be useful for identifying most of the chromosomes that have had no FISH markers.

A combination of FISH and immunostaing revealed three unusual types of chromosomes, a large metacentric chromosome (R1), a small telocentric chromosome (R2) and a minichromosome (M), in some metaphase cells (Fig. 2a). Chromosome R1 had two adjacent but separated centromeres. A subsequent GISH analysis revealed that one arm originated from a S-genome chromosome and the other from a T-genome chromosome (Fig. 2b). Both GRS and NTRS sequences were detected by FISH on the T-genome-derived arm, suggesting that the arm originated from a long arm of chromosome T3. The origin of the S-genome-derived arm remains unclear.

N. sylvestris S-genome signals appeared throughout the small telocentric chromosome R2, suggesting it originated from an S-genome chromosome (Fig. 2b). However, because no FISH signals were detected on the R2 chromosome, the originated chromosome was not able to be identified. Chromosome M was not always detected together with chromosomes R1 and R2, and occasionally appeared as a supernumerary chromosome in a normal karyotype (Fig. 1a, b). Although the size of the chromosome M is very small and is about one quarter that of the smallest of normal tobacco chromosomes, immunostaining with anti-NtCENH3 antibody revealed that the primary constriction (centromere) is located in the middle of the chromosome (Figs. 1c and 2a). GISH analysis suggested that chromosome M originated from an S-genome chromosome (Fig. 1b). However, because no FISH signals were detected on chromosome M, the exact origin remains unknown. Due to its extremely small size, the minichromosome has likely gone undetected in previous studies. The same or similar minichromosomes were observed in different tobacco plants in this study, suggesting the possibility that they are maintained as supernumerary chromosomes in the population and are transmissible from generation to generation. Further studies are needed to classify the minichromosomes into B chromosomes because B chromosomes are not common in Solanaceae, particularly in the genus *Nicotiana* (Jones and Rees 1982).

Acknowledgment This work was supported by the program for the promotion of basic and applied researches for innovations in bio-oriented industry (BRAIN).

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