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Karyotype analysis of *Nicotiana kawakamii* Y. Ohashi using DAPI banding and rDNA FISH

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Abstract Prometaphase cells were used to analyze the karyotype of *Nicotiana kawakamii* Y. Ohashi by means of sequential Giemsa/CMA/DAPI staining and multicolor fluorescence in situ hybridization with 5S and 18S rDNA. Observation of the DAPI-stained prometaphase spreads indicated that *N. kawakamii* had six pairs of large chromosomes, one pair of medium-sized chromosomes and five pairs of small chromosomes. The six pairs of large chromosomes possessed remarkable DAPI bands, and each could be identified from both the DAPI banding pattern and the length of the short arm. The DAPI banding pattern was approximately identical to the CMA and Giemsa banding patterns. Hybridization signals of the 18S rDNA probe were detected on two pairs of large chromosomes. In addition, two pairs of small chromosomes were identified based on the position of the 5S rDNA signals. An idiogram of *N. kawakamii* chromosomes was produced based on DAPI bands and rDNA loci.

Keywords *Nicotiana kawakamii* Y. Ohashi · Karyotype · Giemsa/CMA/DAPI staining · rDNA FISH · Quantitative idiogram

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Introduction

Nicotiana kawakamii Y. Ohashi (2n = 24, unknown genome) is a wild tobacco species discovered in Bolivia in 1968 and classified into the section Tomentosae of the subgenus *Tabacum* on the basis of morphological characters (Ohashi 1976). This species is known to be resistant to potato virus Y (PVY), one of the most serious diseases of Solanaceae crops, including tobacco. Genetic analysis demonstrated that the PVY resistance in *N. kawakamii* is controlled by a recessive gene and that the locus is different from the most common PVY-resistant gene, the *va* locus (Ohashi 1985). The introduction of this character of *N. kawakamii* into cultivated tobacco, *N. tabacum* L., was subsequently attempted (Ohashi 1985).

It has been reported that the chromosome morphology of *N. kawakamii* is similar to that of the core species of Tomentosae (Ogura 1980; Ohashi 1985). However, it is difficult to detect subtle differences in karyotypes of closely related species at metaphase. Recently, prometaphase chromosomes have been shown to be useful material for characterizing the karyotype of closely related species in detail (Fukui et al. 1998; Kitamura et al. 2000). Several banding methods have been developed for mammalian and plant chromosome analyses. One of these is fluorochrome banding with chromomycin A_3 (CMA) and 4′-6-diamidino-2-phenylindole (DAPI) (Schweizer 1976, 1980; Hizume et al. 1983). Two kinds of ribosomal RNA genes (45S and 5S rDNA) have been frequently used as probes for fluorescence in situ hybridization (FISH) to obtain physical markers because of their high copy number (Mukai et al. 1990, 1991; Murata et al. 1997; Fukui et al. 1998).

In the study reported here, the karyotype of *N. kawakamii* was analyzed using prometaphase chromosomes by fluorochrome banding and multicolor FISH with 5S and 18S rDNA probes. A chromosome map reflecting DAPI banding and FISH signal patterns was developed.

Materials and methods

Plant materials and chromosome preparation

N. kawakamii plants were cultivated in a greenhouse maintained at about 25°C under natural light conditions. Vigorously growing root tips were cut and used for karyotype analysis.

Chromosome preparation was as described by Kitamura et al. (1997) with slight modifications. Root tips were pretreated with water at 0°C for 24 h, fixed in 3:1 ethanol:acetic acid at 4°C for 2 days and macerated in 1% Cellulase Onozuka RS (Yakult), 0.75% Macerozyme R-200 (Yakult), 0.15% Pectolyase Y-23 (Seishin Pharmaceutical) and 0.5 m*M* EDTA (Sigma) at 37°C for 10–30 min.

Sequential staining with Giemsa/CMA/DAPI

Chromosome spreads were stained with 2% Giemsa at 25°C for 15 min, washed in distilled water and air-dried. Spreads without overlapping chromosomes were selected and photographed. After de-staining in 70% ethanol, CMA/DAPI staining was carried out according to Kondo and Hizume (1982). Chromosome spreads were stained with 0.1 mg/ml CMA at 25°C for 20 min, briefly rinsed with McIlvaine buffer (citric acid-Na_2HPO_4, pH 7.0), treated with 0.25 mg/ml actinomycin D at 25°C for 20 min, stained with 10 µg/ml DAPI at 25°C for 10 min, washed a few times with distilled water and mounted with 2% DABCO [1,4-diazabicyclo-(2.2.2) octane] in 1:1 glycerol:McIlvaine buffer solution. After 3 days at 4°C in the dark, DAPI and CMA images were obtained by fluorescence microscopy.

Multicolor fluorescence in situ hybridization (McFISH)

The 18S rDNA was amplified from genomic DNA of *N. kawakamii* using primers (5′-AAC GGC TAC CAC ATC CAA GG-3′; 5′-GGT GGT GCC CTT CCG TCA AT-3′) that were designed from 17S rDNA sequences of rice (Takaiwa et al. 1984) and simultaneously labeled with digoxigenin-11-dUTP (Boehringer Mannheim). The 5S rDNA probe was also amplified with a specific primer pair (Kitamura et al. 2000) and labeled with biotin-16-dUTP (Boehringer Mannheim).

The in situ hybridization was performed according to Kitamura et al. (1997) with the following modification: the chromosomal DNA covered by the hybridization mixture, including 100 ng of each probe DNA per slide, was denatured at 80°C for 6 min. The washing temperature after all fluorescence reactions was 37°C. Hybridization signals of the digoxigenin-labeled 18S rDNA probe were amplified with anti-digoxigenin-rhodamine raised in sheep (Boehringer Mannheim) and detected by Texas red-labeled anti-sheep-IgG (Vector Laboratories). The signals of the biotinylated 5S rDNA probe were detected with fluorescein isothiocyanate (FITC)-avidin (Vector Laboratories). Chromosomal DNA was counterstained with 10 µg/ml of DAPI.

Hybridization images and DAPI images were taken independently with a high-sensitive, cooled CCD camera (PXL 1400, Photometrics), and merged with IPLab Spectrum software.

Image analysis

Five DAPI-stained prometaphase spreads of *N. kawakamii* were used for image analysis. A quantitative idiogram was established by CHIAS III (Kato and Fukui 1998) using a public domain program, NIH image. Image analysis was performed according to Kato et al. (1997).

A single prometaphase spread after sequential Giemsa/CMA/DAPI staining was used for comparative analysis of the banding patterns.

Results and discussion

One pair of satellite chromosomes was found in the karyotype of *N. kawakamii* (Fig. 1). Another chromosome pair possessed a satellite-like structure in the terminal region of the short arm that was sometimes observed at prometaphase (Fig. 2). However, this structure never separated from the short arm, while the satellite of the regular satellite chromosome frequently separated from the short arm at prometaphase. Therefore, *N. kawakamii* has only one pair of true satellite chromosomes. This differs from Ogura (1980) who observed two pairs of satellite chromosomes.

The metaphase chromosome complement of *N. kawakamii* showed clear karyotypic bimodality consisting of large subtelocentrics and small meta- to submetacentrics (Ogura 1980) as shown in the core species of

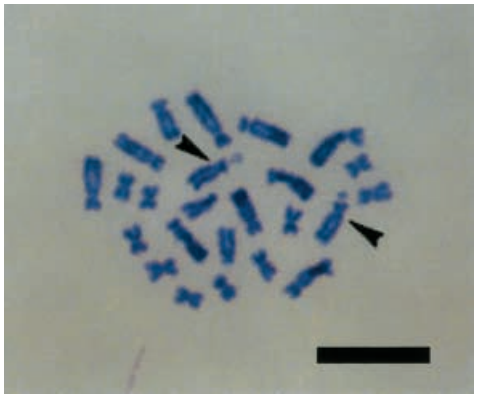

Fig. 1 Giemsa-stained metaphase spread of *N. kawakamii*. *Arrowheads* indicate the satellite chromosomes. *Bar*: 10 µm

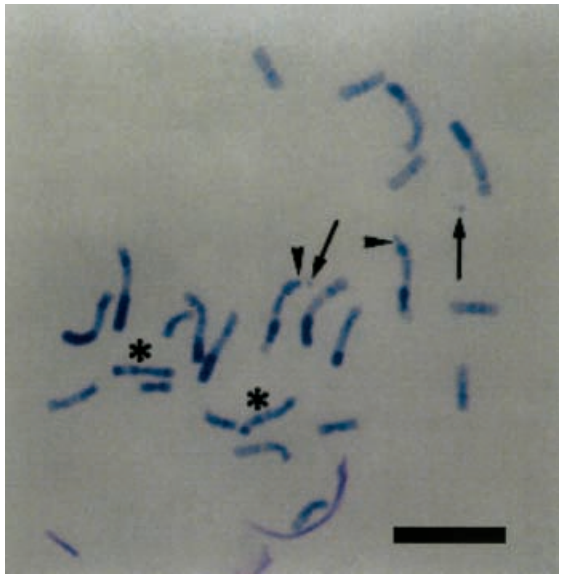

Fig. 2 Giemsa-stained prometaphase chromosomes of *N. kawakamii*. *Arrows*, *asterisks* and *arrowheads* indicate satellites, satellite chromosomes and satellite-like structures, respectively. *Bar*: 10 µm

Tomentosae (Goodspeed 1954, Kitamura et al. 2000). In prometaphase spreads, some chromosomes exhibited conspicuous DAPI bands (Fig. 3), which are difficult to detect in metaphase chromosomes. On the basis of their size, the chromosomes of *N. kawakamii* were classified into six pairs of large chromosomes, one pair of medium-sized chromosomes and five pairs of small chromosomes. The number of small chromosomes is the same as that reported by Ohashi (1976, 1985).

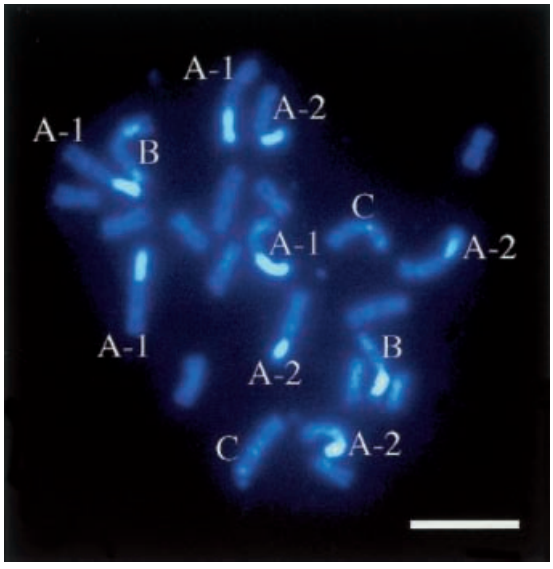

Fig. 3 DAPI-stained prometaphase spread of *N. kawakamii*. *Bar*: 10µm. *A-1, A-2, B* and *C* are explained in the text

DAPI bands were detected on all large chromosomes (Fig. 3). The large chromosomes could be divided into three groups; group-A chromosomes had DAPI bands in the terminal region of the long arm, group-B chromosomes had bands both in the terminal region of the short arm and in the subterminal region of the long arm, and group-C chromosomes had bands in the terminal and intercalary regions of the long arm. The chromosomes of group A could be further subdivided into group A-1 and group A-2 based on the size of the DAPI banding region. Therefore, the six pairs of large chromosomes could be classified into two pairs of group A-1, two pairs of group A-2, one pair of group B and one pair of group C (Fig. 6).

No remarkable DAPI band was detected on the remaining chromosomes, and the morphology of the small chromosomes was very similar. It has been reported that the DAPI banding pattern does not correspond to the CMA banding pattern in some species (Doudrick et al. 1995; Matsuyama et al. 1996). Therefore, an attempt was made to discriminate between the small chromosomes of *N. kawakamii* by Giemsa/CMA/DAPI banding. The CMA (Fig. 4A), DAPI

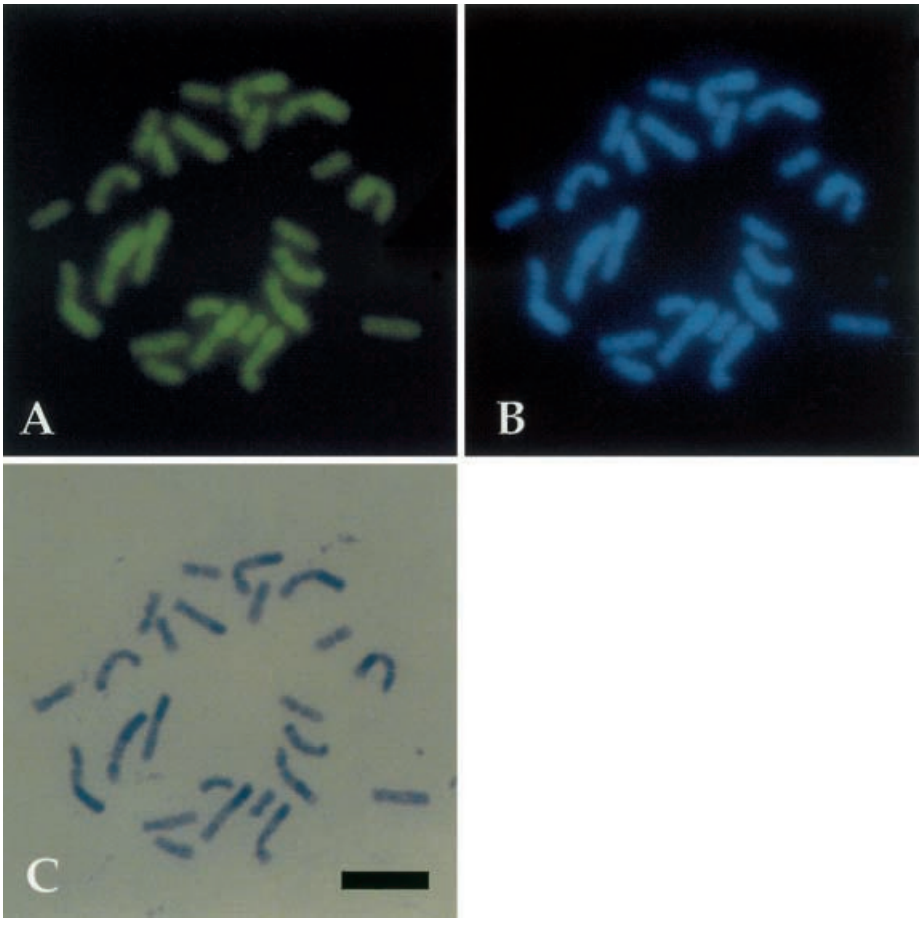

Fig. 4A–C Prometaphase chromosomes of *N. kawakamii* after CMA (**A**), DAPI (**B**) and Giemsa banding (**C**). *Bar*: 10 µm

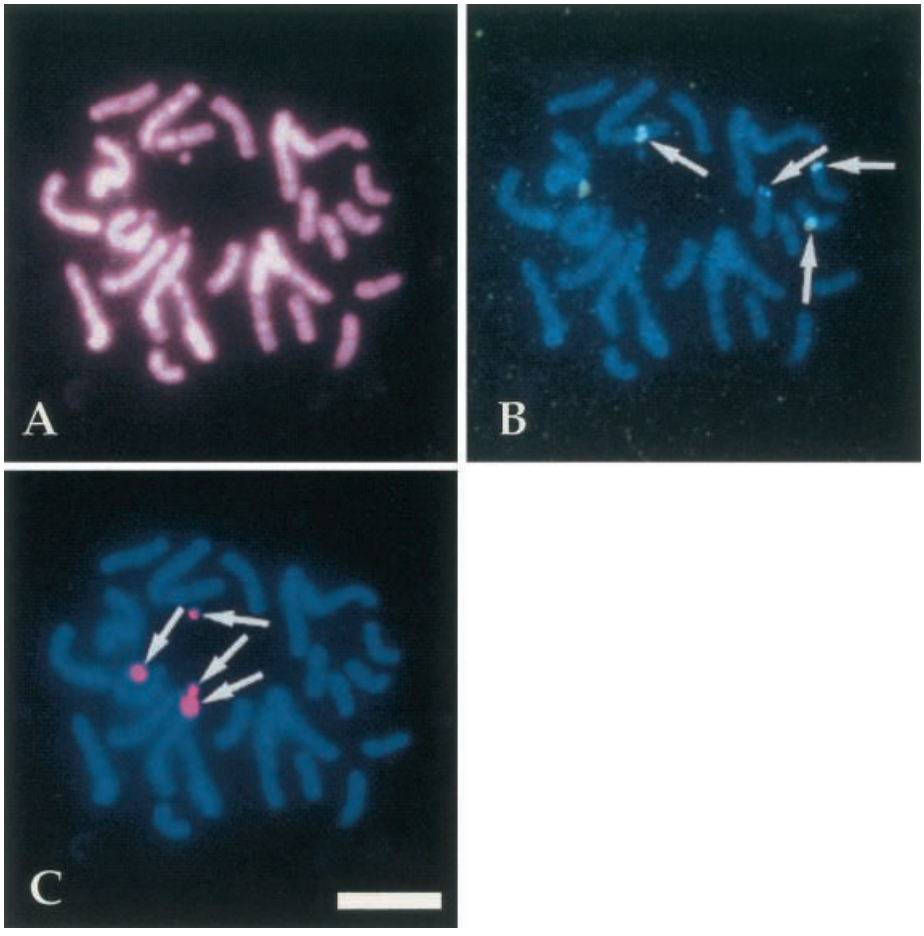

Fig. 5A–C Multicolor FISH with 5S and 18S rDNA probes in prometaphase chromosomes of *N. kawakamii*. **A** DAPI banding, **B** FISH with 5S rDNA (*arrows*), **C** FISH with 18S rDNA (*arrows*). *Bar:* 10 μm

Table 1 Chromosome measurements from firve prometaphase images of *N. kawakamii*

Chromosome number	Size	DAPI pattern	Length of short arm (μm) [a]	Length of long arm (μm) [a]
Chromosome 1	Large	A-1	3.54 ± 0.11	10.34 ± 0.23
Chromosome 2	Large	A-1	2.07 ± 0.10	11.12 ± 0.23
Chromosome 3	Large	A-2	1.86 ± 0.06	10.60 ± 0.28
Chromosome 4	Large	A-2	2.73 ± 0.06	9.75 ± 0.16
Chromosome 5	Large	B	3.60 ± 0.19	9.67 ± 0.21
Chromosome 6	Large	C	2.35 ± 0.09[c]	9.55 ± 0.14
Chromosome 7	Medium		1.85 ± 0.03	8.25 ± 0.26
Chromosome 8	Small		2.86 ± 0.08	3.74 ± 0.11
Chromosome 9	Small		2.79 ± 0.12	4.15 ± 0.14
Unidentified [b]	Small		2.74 ± 0.08	3.37 ± 0.10

[a] Average value ± SE (standard error)
[b] Average length of three non-identifiable small metacentrics
[c] Satellite regions were excluded from the calculation

(Fig. 4B) and Giemsa (Fig. 4C) banding patterns were all similar. Even image analysis for a chromosomal spread sequentially stained with CMA, DAPI and Giemsa (Fig. 4), revealed no differences in the size and location of positively stained regions among the three staining patterns.

Figure 5 shows FISH signals obtained with 5S and 18S rDNA probes in prometaphase chromosomes of *N. kawakamii*. Hybridization with the biotinylated 5S rDNA probe was visualized as yellow-green. Four hybridization signals were detected (Fig. 5B). These occurred on two pairs of small chromosomes; One was localized on the proximal region of the long arm, and the other was in the terminal region of the short arm. Thus, two out of five small chromosome pairs were identified. The signal intensity of the proximal site was stronger than that of the terminal one, suggesting a difference in the copy number of the 5S rRNA genes (Murata et al. 1997).

As shown in Fig. 5C, four strong 18S rDNA hybridization signals were detected on two pairs of large chromosomes. These were mapped onto the satellite region

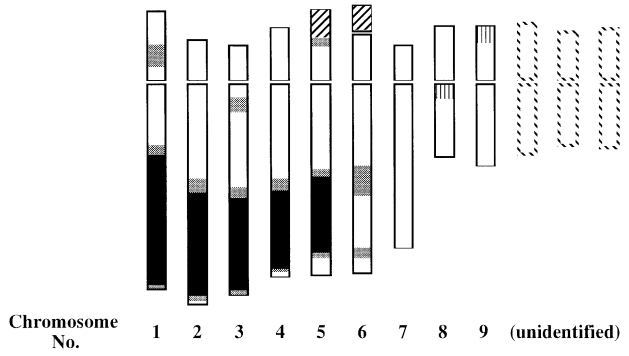

Fig. 6 Quantitative idiogram of DAPI-stained prometaphase chromosomes of *N. kawakamii. White*, *gray* and *black* regions indicate weak, intense and the most intense fluorescence with DAPI, respectively. ▨ and ▥ indicate FISH signals of 18S and 5S rDNA probes, respectively. Chromosomes 5 and 6, which harbor 18S rDNA loci, correspond to chromosomes 4 and 3 in Lim et al. (2000), respectively

of the group-C chromosome pair and the terminal region of the short arm of the group-B chromosome pair.

Using five prometaphase spreads possessing a similar degree of condensation, we measured and processed chromosomal lengths through the chromosome analyzing system CHIAS III (Table 1). Based on the difference of the length of their short arms each pair of group A-1 and group A-2 could be identified. Therefore, with the help of image analysis using DAPI-stained prometaphase spreads, all of the large chromosomes were identified.

Based on the five DAPI-stained prometaphase spreads (Table 1), an idiogram was developed (Fig. 6). Nine out of twelve pairs of *N. kawakamii* chromosomes could be identified. Chromosomes 1 and 2 belong to group A-1, with the length of the short arm of chromosome 1 being longer than that of chromosome 2. Chromosomes 3 and 4 belong to group A-2, with the length of the short arm of chromosome 4 being longer than that of chromosome 3. Chromosome 5 belongs to group B and harbors 18S rDNA but no satellite. Chromosome 6 belongs to group C and has the 18S rDNA within the satellite. Chromosome 7 is the only medium-sized chromosome. Chromosomes 8 and 9 have the 5S rDNA in the proximal region of the long arm and the terminal region of the short arm, respectively.

These results enable us to analyze relationships between *N. kawakamii* and other Tomentosae species at the chromosomal level. Recently, the distribution of DAPI bands and rDNA sites in *N. tomentosiformis* Goodspeed and *N. otophora* Grisebach (both 2n = 24, TT), both closely related to *N. kawakamii*, was reported (Kitamura et al. 2000). Comparison of their idiograms with Fig. 6 of this study suggests that the karyotype of *N. kawakamii* is more similar to that of *N. otophora* than that of *N. tomentosiformis*. In spite of this, highly repeated DNA sequences present in *N. kawakamii* have not been found in *N. otophora* (Matyásek et al. 1997).

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