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Development of a Method to Produce Chromosome Lacking Lines (CLLs) in *Nicotiana tabacum* L. "Red Russian"

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Abstract

Monosomic lines of *Nicotiana tabacum* are helpful to confirm the location of genes on specific chromosomes. In the cross *N. nudicaulis* and *N. tabacum*, hybrid seedlings express lethal symptoms, which are controlled by the S subgenome of *N. tabacum*. To identify the responsible chromosome, we needed to produce chromosome lacking lines (CLLs) of *N. tabacum* L. "Red Russian" and use them to cross with *N. nudicaulis*. From a cross of (*N. tabacum* × *N. tomentosiformis*) × *N. tabacum*, 380 BC1 individuals were obtained. Using a Haplo-Q line (a monosomic line lacking the single linkage group 11) and *N. tabacum*, we found that qPCR is a simple and reliable screening method for CLLs of *N. tabacum*. The marker PT30342 is located on linkage group 11, and the ΔCt value (Ct Actin - Ct PT30342) was 2.0 for a disomic line and was 1.097 for a Haplo-Q line. By the use of flow cytometry, qPCR and chromosome counting together as a screening method, we identified 6 CLLs lacking 2 to 6 chromosomes. Compared with conventional methods, our method is a rapid technique for making and screening CLLs ofthe S or S/T subgenome of *N. tabacum*. Further, these CLLs will be useful to identify the location of two or more factors on chromosomes controlling a variety of genetic problems affecting breeding. Here, we only made CLLs of the S or S/T subgenome of *N. tabacum*. We will use the method established in this study to produce CLLs of the T subgenome of *N. tabacum*, and gather a full set of CLLs of *N. tabacum*. qPCR could also be applied to the identification of chromosome aberrations in other plants.

Keywords

Chromosome Lacking Lines, Quantitative Real-Time Polymerase Chain Reaction, Simple Sequence Repeat Marker, Flow Cytometry, *Nicotiana*

tabacum L.

1. Introduction

Hybrid lethality is an important obstacle in plant breeding programs, and in *Nicotiana*, is expressed as five specific phenotypes depending on the cross combination [1] [2]. Among *Nicotiana* species, only *N. tabacum* (2n = 48, SSTT) and *N. rustica* L. (2n = 48, PPUU) are cultivated, whereas the others are wild species. Of the two cultivated species, hybrid lethality is mainly exhibited in combinations between *N. tabacum* and wild tobacco species, and has been well studied in crosses between species of section *Suaveolentes* and *N. tabacum* [3].

N. tabacum is a natural allotetraploid belonging to section *Genuinae* that originated by interspecific hybridisation of *N. sylvestris* Spegazzini & Comes (2n = 24, SS) with *N. tomentosiformis* Goodspeed (2n = 24, TT), and subsequent chromosome doubling [4]-[11]. Each chromosome of *N. tabacum* is lettered alphabetically (A to Z, excluding X and Y); chromosomes A through L belong to the T subgenome and M through Z to the S subgenome. A complete set of 24 monosomic lines of *N. tabacum* (Haplo-A to Z) has been established in the genetic background of "Red Russian" [12] [13] [14] and is useful for locating genes on specific chromosomes. Previously, monosomic analyses have identified the Q chromosome of the S subgenome as carrying a gene or genes that trigger hybrid lethality in crosses between section *Suaveolentes* and *N. tabacum* [3].

When *N. nudicaulis* Watson, belonging to the section *Repandae* [11] [15], is crossed with *N. tabacum*, hybrid seedlings show lethal symptoms [1] [16], which are controlled by the S subgenome of *N. tabacum* [17]. However, this hybrid lethality is different from that expressed in crosses between section *Suaveolentes* and *N. tabacum*, whose lethality is not related to the Q chromosome [17]. Therefore, additional monosomic lines lacking each chromosome the S subgenome of *N. tabacum* are needed to determine the causative chromosome. Unfortunately, these other monosomic lines for these subgenomes of *N. tabacum* no longer seem to be available. Thus, we needed to produce new monosomic lines belonging to the S subgenome of *N. tabacum*.

Olmo [12] described how monosomic lines of the S subgenome were isolated by design from crosses between *N. tabacum* and *N. tomentosa* Ruiz & Pavon followed by recurrent backcrossing to *N. tabacum*. Clausen and Cameron [13] used chromosome analysis and morphological characterisation to detect monosomic lines of *N. tabacum*. However, this method is time consuming and easy to misinterpret, because morphological characters are sometimes changed depending on the weather or cultivation conditions.

In humans, chromosome aneuploidy is a major cause of foetal morbidity, and leads to neonatal mortality. Currently, prenatal diagnosis is based on chromosome analysis of foetal cells derived from an amniocentesis or chorionic villi

sample. Recently, methods to detect chromosome aneuploidy by quantitative real-time PCR (qPCR) have been reported, which enable accurate diagnosis of chromosome aneuploidy within a few hours [18] [19] [20]. In *N. tabacum*, the first linkage map was constructed by Bindler *et al.* [21] using simple sequence repeat (SSR) markers. In this linkage map, eleven linkage groups are assigned to the S genome and four linkage groups to both the S and T genome (S/T genome). Using specific markers mainly grouped in different parts of the respective linkage groups, we aimed to develop a rapid technique for making and screening chromosome lacking lines (CLLs) of the S or S/T subgenome of *N. tabacum*.

Monosomic lines originate from an imbalance of chromosomal pairing. In many cases, polyploid organisms, chromosome fragments, or structural chromosomal rearrangements appear unexpectedly in monosomic progeny [22] [23] [24] [25]. Some BC1 plants have higher or lower chromosome numbers than parental lines, so to efficiently select CLLs that spontaneously appear, we carried out flow cytometry to analyse DNA content before qPCR as a new screening method for CLLs of the S or S/T subgenome of *N. tabacum*.

2. Methods

2.1. Plant Materials

Seeds of *N. tabacum* L. "Red Russian" (2n = 48, SSTT) and *N. tomentosiformis* Goodspeed (2n = 24, TT) were supplied by Japan Tobacco Inc. (Oyama, Japan). The Haplo-Q (2n = 47), *N. tabacum* monosomic line was supplied by Dr. T. Kubo, Japan Tobacco, Inc. A haploid of *N. tabacum,* which was induced through anther culture and maintained in our laboratory, was used as an internal standard in flow cytometry. Monosomic lines of *N. tabacum* were originally produced in the Department of Genetics, University of California, Berkeley [14]. All plants were cultured and retained in the greenhouse (natural day length) of Meiji University.

2.2. Cross of (*N. tabacum* × *N. tomentosiformis*) × *N. tabacum*

To obtain BC1 lines, Clausen and Cameron [13] carried out the cross (*N. tabacum* × *N. tomentosa*) × *N. tabacum* by conventional crossing. However, considerable evidence suggests that the paternal progenitor of *N. tabacum* is *N. tomentosiformis* [4]-[11]. So, F1 plants of *N. tabacum* × *N. tomentosiformis*were used for backcrosses with *N. tabacum* in the present study.

Since it is difficult to obtain BC1 seedlings from (*N. tabacum* × *N. tomentosiformis*) × *N. tabacum* using conventional crosses, ovule culture was carried out 9 - 12 days after conventional pollination and seedlings were obtained as described by Marubashi and Nakajima [26]. Flowers of F1 plants from the cross between *N. tabacum* and *N. tomentosiformis* were emasculated before flowering and pollinated with fresh *N. tabacum* pollen. Nine to 12 days after pollination, cross-pollinated flowers of F1 from the cross between *N. tabacum* and *N. tomentosiformis* were collected and the sepals, petals, and styles were removed.

The ovaries were surface-sterilised with 70% ethanol for 30 s with 5% sodium hypochlorite for 10 min, then rinsed three times with sterilised water. The ovary walls were peeled to expose the placenta with intact ovules. All of the ovules were excised and cultured on Petri dishes containing 8 ml 1/2x MS medium [27] supplemented with 3% sucrose and 0.25% Gelrite, pH 5.8, at 28˚C under continuous illumination (30 mmol photons $s^{-1} \cdot m^{-2}$).

2.3. Cultivation of BC1 Seedlings

Immediately after germination, all viable BC1 seedlings from crosses (*N. tabacum* × *N. tomentosiformis*) × *N. tabacum* were separately transferred onto the surface of 1/2x MS medium with 1% sucrose at 28˚C under continuous illumination (123 $mmol \cdot s^{-1} \cdot m^{-2}$ or 147 $mmol \cdot s^{-1} \cdot m^{-2}$). At 30 DAG, these BC1 seedlings were potted and cultivated on a culture shelf in the laboratory.

2.4. Crosses between Haplo-Q and *N. tabacum* L. "Red Russian"

Flowers of Haplo-Q, used as maternal parents, were emasculated and then pollinated with pollen of *N. tabacum* L. "Red Russian" by conventional crossing. F1 plants were cultivated in the greenhouse under natural lighting conditions.

2.5. DNA Extraction

Genomic DNA of Haplo-Q, *N. tabacum* and F1 plants of Haplo-Q × *N. tabacum* was extracted from leaves of individual plants using the cetyltrimethylammonium bromide method of Murray and Thompson [28]. Genomic DNA of BC1 and *N. tabacum* as control for qPCR was extracted from leaves of 40- to 50-day-old seedlings using a Plant DNA Isolation Reagent (TaKaRa Bio Inc., Shiga, Japan); these BC1 seedlings were screened after flow cytometry.

2.6. Determination of SSR Markers on Each Linkage Group Belonging to the S or S/T Subgenome for qPCR

SSR markers for 15 linkage groups belonging to the S or S/T genome were quoted from the report by Bindler *et al.* [21]. Genomic DNA of N. tabacum was used as template for PCR. The reaction mixture contained 0.25 μl (5 U/μl) TaKaRa Ex Taq, 5 μl 10× Ex Taq Buffer (20 mM Mg2+ plus), 4 μldNTP mixture (2.5 mM, TaKaRa Bio Inc.), 0.5 μM primer, and 50 ng DNA in a total volume of 50 μl. PCR was performed using a 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA) programmed for 2 min at 98˚C for initial denaturation, followed by 30 cycles of 10 s at 98˚C, 30 s at 55˚C, 30 s at 72˚C, and a final extension of 1 min at 72˚C. PCR products were separated by electrophoresis on 1.5% agarose gels in TAE buffer and stained with ethidium bromide to visualise DNA bands.

2.7. qPCR

In each qPCR experiment, genomic DNA from a disomic line (*N. tabacum* L.

"Red Russian") constituted a control. For an internal DNA quality control, transcripts were amplified from the housekeeping marker for one pair actin marker (Actin), and a no-template control (H_2O) was also included in each experiment. In qPCR experiments distinguishing between Haplo-Q and *N. tabacum*, the reaction mixture contained 25 µl KOD SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan) 1 µl 50× ROX reference dye (Toyobo Co., Ltd), 0.5 µM primer, and 50 ng DNA in a total volume of 50 µl; qPCR was performed using a 7300 real-time PCR system (Applied Biosystems). In qPCR experiments between BC1 and *N. tabacum*, the reaction mixture contained 25 µl KOD SYBR qPCR Mix, 0.5 µM primer, and 50 ng DNA in a total volume of 50 µl; qPCR was performed using a real-time PCR system (LightCycler Nano; Roche, Basel, Switzerland).

The threshold cycle (Ct) values for Actin and each target marker were determined for each sample as the number of cycles at which fluorescent emission first exceeded the baseline value. Differences in the Ct values between Actin and each target marker (ΔCt = (Ct Actin - Ct target marker)) were then calculated for each sample as an indicator of the number of copies of the chromosome that each target marker located in the genomic DNA.

2.8. Chromosome Analysis

Root tips of viable seedlings were pretreated with distilled water for 24 h at 4˚C and with 2 mM 8-hydroxyquinoline for 4 h at 4˚C, then fixed in a 3:1 mixture of ethanol and acetic acid overnight to determine chromosome numbers. The root tips were hydrolysed in 1 N HCl for 10 min at 60˚C, stained with Schiff's reagent and squashed in 45% acetic acid. The number of chromosomes in at least five root tip cells for each plant was counted under a BX51 light microscope (Olympus, Tokyo, Japan) and photographed using a DP70 automatic photomicrography system (Olympus).

2.9. Analysis of Pollen Fertility

To determine pollen fertility, pollen grains were mixed thoroughly with acetocarmine stain and observed under the BX51 light microscope, then photographed using the DP70 automatic photomicrography system. Darkly stained pollen grains were recorded as fertile and viable, and unstained or very lightly stained ones were counted as sterile or inviable. For 14 plants, 1500 pollen grains were collected from at least three flowers to calculate the percentage of fertile pollen from each plant.

2.10. Flow Cytometry

For cytometric analysis, nuclei were isolated from 100 mg leaves (except for midrib) of BC1 seedlings from crosses of (*N. tabacum* × *N. tomentosiformis*) × *N. tabacum* cultivated in the laboratory; the leaves were chopped off and macerated in ice-cold buffer [29]. Each sample was analysed twice. The macerated tissue was filtered through a 25 mm nylon mesh. Nuclei were collected from the filtrate

by centrifugation (5 min at 3000 rpm and 4˚C) and suspended in ice-cold buffer supplemented with 5 mg/ml DAPI for 1 min at 4˚C. The DNA content of the isolated nuclei was analyzed on a flow cytometry system (Cell Lab Quanta SC; Beckman Coulter Inc., Brea, CA, USA); 22,000 nuclei or less than 22,000 nuclei were counted.

3. Results

3.1. Cross of (*N. tabacum* × *N. tomentosiformis*) × *N. tabacum*

From the method of Clausen and Cameron [13], monosomic lines were obtained from the cross of (*N. tabacum* × *N. tomentosa*) × *N. tabacum* by conventional crossing. Because *N. tomentosiformis* is the paternal progenitor of *N. tabacum* [4]-[11], F1 plants were obtained from the conventional cross of *N. tabacum* × *N. tomentosiformis*. Crossing of (*N. tabacum* × *N. tomentosiformis*) × *N. tabacum* was carried out via conventional cross-pollination. Because falling of blossoms occurred two weeks after cross-pollination and no BC1 seedlings were obtained, ovule culture was carried out to obtain BC1 seedlings.

Therefore, 1947 flowers of F1 of *N. tabacum* × *N. tomentosiformis* were pollinated with fresh *N. tabacum* pollen by conventional cross-pollination. From 1031 ovaries, 11,644 fertilised ovules were obtained 9 - 12 days after pollination, and 380 seeds began to germinate 1 month after pollination; all of these BC1 seedlings were left at 28˚C (Table 1).

3.2. Selection of SSR Markers on Each Chromosome of the S or S/T Subgenome for qPCR

A set of 140 pairs of SSR primers on the 15 linkage groups belonging to the S or S/T genome were taken from Bindler *et al.* [21], enabling amplification of 100 - 300 base-pair fragments. Of these SSR markers, 15 sets of SSR markers specific for 15 linkage groups belonging to the S or S/T subgenome were detected by PCR. A 143 base-pair fragment Actin (accession number GQ339768.1) was used as a control. These markers were used in selection of a suitable primer set for qPCR. Complete information for the primers is in Table 2 and Figure 1.

3.3. qPCR Analysis in a Disomic and a Haplo-Q Line

In order to show that qPCR is a simple and reliable screening method for CLLs of *N. tabacum*, a specific SSR marker of linkage group 11 (Q chromosome) was used in qPCR; Tezuka *et al.* [30] reported that the Q chromosome corresponds

Table 1. Backcross of (*N. tabacum* × *N. tomentosiformis*) × *N. tabacum*.

Cross combination	No. of Flowers Pollinated	No. of Ovaries with Ovules	No. of Ovules Cultured	No. of Hybrids Obtained	No. of Hybrids for qPCR	Chromosome Lacking Lines
(*N. tabacum* × *N. tomentosiformis*) × *N. tabacum*	1947	1013	11644	380	103*	7

*26 of the hybrids died before qPCR.

Table 2. Assay information for SSR markers mapping to all linkage groups (LG) belonging to the S subgenome or S/T subgenome from Bindler *et al.* [21]. For each marker, the marker name, the two primer sequences, and the expected fragment size are described. Annealing temperatures are shown in Methods.

Name described in Bindler *et al.* [21]	Forward Primer	Reverse Primer	Size (bp)
Actin	AGTCCTCTTCCAGCCATCCA	AAGCCGTGATTTCCTTGCTC	143
PT30157 (LG1)	AATCTTCGAGCAGAAATCCG	TGTCCTTCCATTTAGATTCCAGA	230
PT30025 (LG3)	TGGAGGAACCAACAAGGAAG	TGGAGGAACCAACAAGGAAG	203
PT30111 (LG5)	AGCCAGCCACCAAATTTATC	GGAACATTGCTCAAGCCCTA	208
PT30368 (LG6)	TCACCAATTATGCCGATTCA	CACACACTTGTTTGGAATTGAGA	142
PT30411 (LG7)	AACGCGCGTATCGTAAGACT	CAAATCACAATCAAGAAATTTGAG	204
PT30307 (LG8)	AAAGAAGCACGGTCAAATAGG	GCAACAACAAGGTGTCATGG	222
PT30235 (LG9)	TTTCAGCCGTCACCTCTTCT	AGCGATGCAACAATGATGAG	171
PT30241 (LG10)	AAGTCTCGTGTGGTTGCTTT	AAAGGGCAATGTGTCTAGCTC	199
PT30342 (LG11)	GACAACAATCAGTAAAGGAAACGA	AATGCAAGACCCTGTCAACC	227
PT1313 (LG13)	GTGGATTGGTTTACACGC	CTCTGTCTGCCAAATTGC	210
PT30302 (LG16)	CCTTCCTAACCTCAGCTGGAA	TATGCCAATGCTTCTTGTGG	222
PT30097 (LG18)	GCCGCAACTAAATTCTCCAT	GGAGTCCGCAAGAGAGGAAT	186
PT40010 (LG20)	GCGTGAAGCAACTAGAGAGAGA	CCATCCATTGCTGCTGATAC	188
PT30186 (LG21)	GAGCCAGAAACAGAGCAAGAA	GATGAATAGCTCTGAATGCTGC	230
PT30324 (LG22)	TGCTCTGCGTTAGAACAGGA	CGACGAGAGAAGATTAGTGAAAGA	151

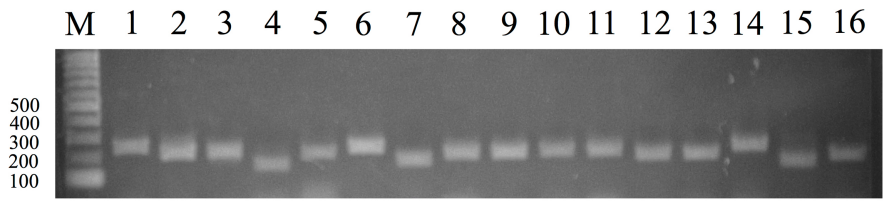


Figure 1. Detection of 15 sets SSR markers. These 15 sets SSR marker map to chromosomes belonging to the S or S/T genome in *N. tabacum* L. "Red Russian". M: size marker OneSTEP Ladder 1 kbp (1 - 10 kbp), Lanes 1 - 15: SSR markers mapping to linkage groups 1, 3, 5, 6, 7, 8, 9, 10, 11, 13, 16, 18, 20, 21, and 22 of *N. tabacum* L. "Red Russian", respectively (Bindler *et al.* [21]). Unit of the molecular length shown in **Table 2**. Lane 16: Actin as a control in *N. tabacum* L. "Red Russian".

to linkage group 11 in the linkage map of Bindler *et al.* [21]. Ct values between expression of Actin and PT30342 (ΔCt) were validated in samples of genomic DNA from both a disomic line (*N. tabacum*) for a control and a Haplo-Q line confirmed by chromosome analysis; the marker PT30342 is located on linkage group 11, which was confirmed by PCR. The difference in ΔCt (Ct Actin - Ct PT30342) for a disomic was 2.0 and for a Haplo-Q line was 1.097 (**Figure 2**). This difference in ΔCt between a disomic and a Haplo-Q line proved that qPCR analysis can distinguish the CLLs from the BC1 line.

To compare the qPCR and conventional methods, genomic DNA of 14 F1

plants from Haplo-Q × *N. tabacum* L. "Red Russian" and genomic DNA of a disomic for a control were analysed by qPCR, chromosome analysis and fertile pollen percentage. In qPCR, 11 Haplo-Q, with a value of ΔCt between 1.23 and 1.57, and three disomics, with a value of ΔCt between 1.83 and 3.17, were identified. Histograms illustrating the mean values of this ratio are shown in Figure 3. The 14 F1 plants were evaluated by chromosome analysis. These results agreed with those of qPCR (Table 3 and Figure 4).

Empirically, in F1 plants of Haplo-Q × *N. tabacum* with a pollen fertility percentage lower than 50%, Haplo-Q is likely included. Using this knowledge, analysis of fertile pollen percentage was carried out in 14 F1 plants. In contrast to results of qPCR and chromosome analysis, the pollen fertility of one disomic was low, and a high percentage of fertile pollen was observed in the majority of Haplo-Q samples (Table 3 and Figure 5).

These results show that when the screening method was based on the percentage of fertile pollen as assessed by acetocarmine staining, the interpretation

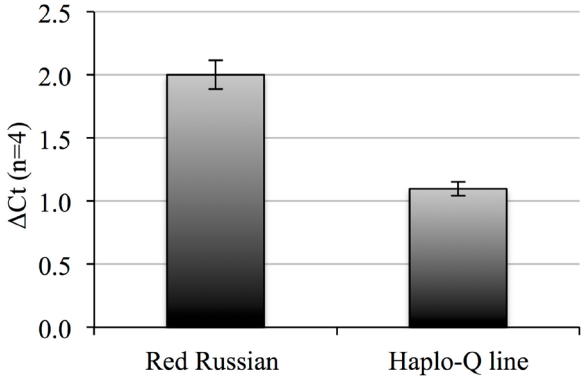


Figure 2. ΔCt of an SSR marker in a disomic (*N. tabacum* L. "Red Russian") and a Haplo-Q line. ΔCt was defined as (Ct Actin - Ct PT30342). The SSR marker was PT30342.

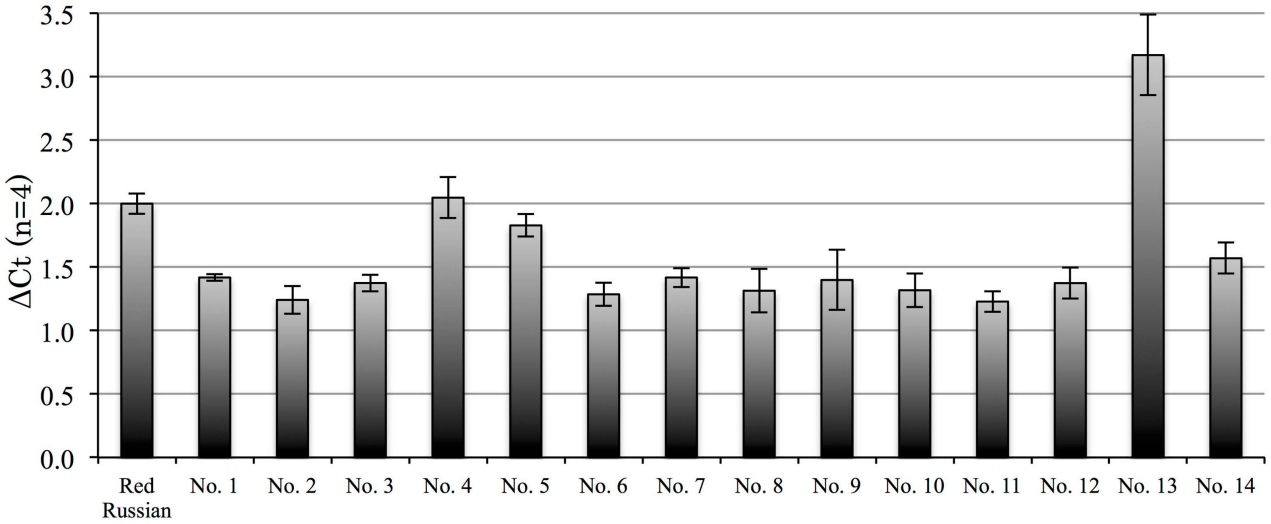


Figure 3. ΔCt of an SSR marker in a cross between Haplo-Q and *N. tabacum* L. "Red Russian". Fourteen F1 plants were used.

Table 3. Analysis of chromosomes and percentage of fertile pollen.

Line No.	Number of Chromosomes (2n)	Fertile Pollen Percentage (%)
1	47	91.7
2	47	45.0
3	47	89.6
4	48	92.3
5	48	93.8
6	47	79.2
7	47	82.5
8	47	85.4
9	47	64.8
10	47	20.4
11	47	76.1
12	47	83.2
13	48	59.5
14	47	82.5
"Red Russian"	48	95.6

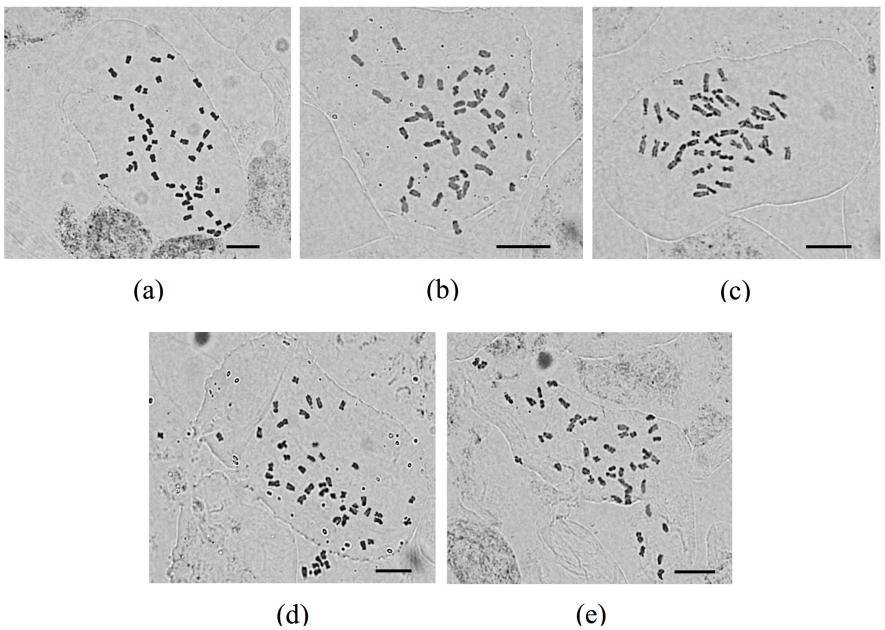


Figure 4. Chromosome number in a cross between Haplo-Q and *N. tabacum* L. "Red Russian". Images of root tip cells of tobacco plants, showing the number of chromosomes. (a) *N. tabacum* (control, 2n = 48); (b) No. 13 F1 plant (2n = 48); (c) No. 10 F1 plant (2n = 47); (d) No. 11 F1 plant (2n = 47). (e) No. 14 F1 plant (2n = 47).

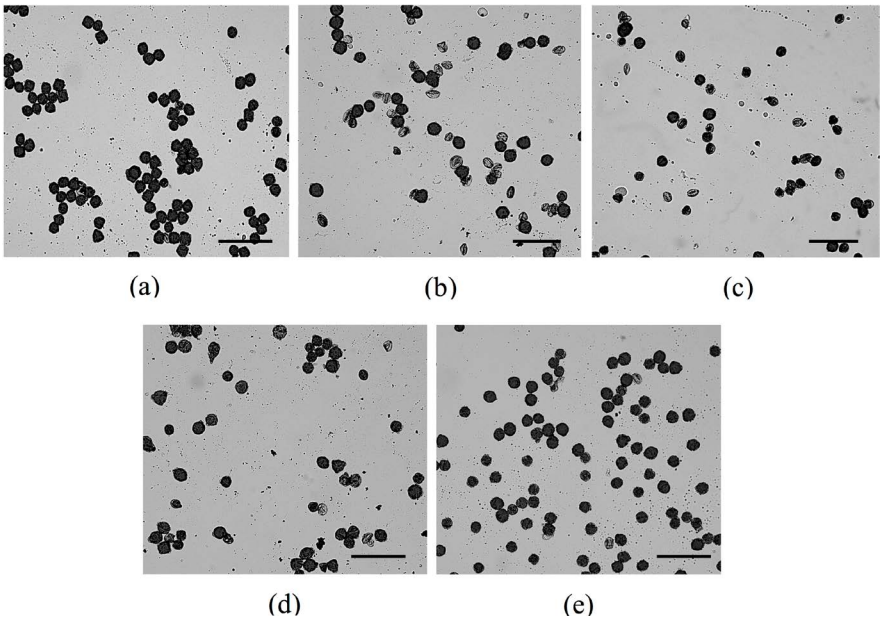


Figure 5. Pollen fertility in a cross between Haplo-Q and *N. tabacum* L. "Red Russian". Fourteen F1 plants were used. Darkly stained pollen grains were recorded as viable and fertile, and unstained or very lightly stained ones were counted as sterile or inviable. (a) Image of pollen grains of *N. tabacum* as a control, showing the fertility of pollen grains; (b) image of pollen grains of No. 13 F1 plant, showing the fertility of pollen grains; (c) image of pollen grains of No. 10 F1 plant, showing the fertility of pollen grains; (d) image of pollen grains of No. 11 F1 plant, showing the fertility of pollen grains; (e) image of pollen grains of No. 14 F1 plant, showing the fertility of pollen grains. Scale bars: 20 μm.

would be a misclassification. In addition, this result demonstrates that morphological characterisation is not suitable as a method for the selection of CLLs.

3.4. Flow Cytometric Analysis

To remove BC1 plants with higher or lower chromosome numbers than 48 (DNA content) from the samples used next for qPCR, flow cytometry was carried out on 380 BC1 plants. Samples of leaf cells of a haploid of *N. tabacum* and of normal *N. tabacum* as the internal standards. The G1 peak of haploid *N. tabacum* appeared at a fluorescence value of 150, and that of *N. tabacum* appeared at a fluorescence value of 300 (**Figure 6**). In 380 BC1 plants, some had a G1 peak with a fluorescence value less than that of the haploid of *N. tabacum* or more than that of *N. tabacum* (**Figure 7(a)** and **Figure 7(b)**). In addition, chimeric plants were also included in these 380 BC1 plants (**Figure 7(c)**). These BC1 plants were excluded from the samples of qPCR analysis.

In a preliminary experiment, the G1 peaks of BC1 plants fell between the fluorescence values of 250 and 300, and many of these plants had 40-48 chromosomes based on qPCR (data not shown), so these plants were selected. Using cytometric analysis, 103 BC1 plants were selected with a G1 peak fluorescence value of 250 - 300 as samples for follow-up qPCR analysis (**Figure 7(d)** and **Figure 7(e)**; **Table 1**).

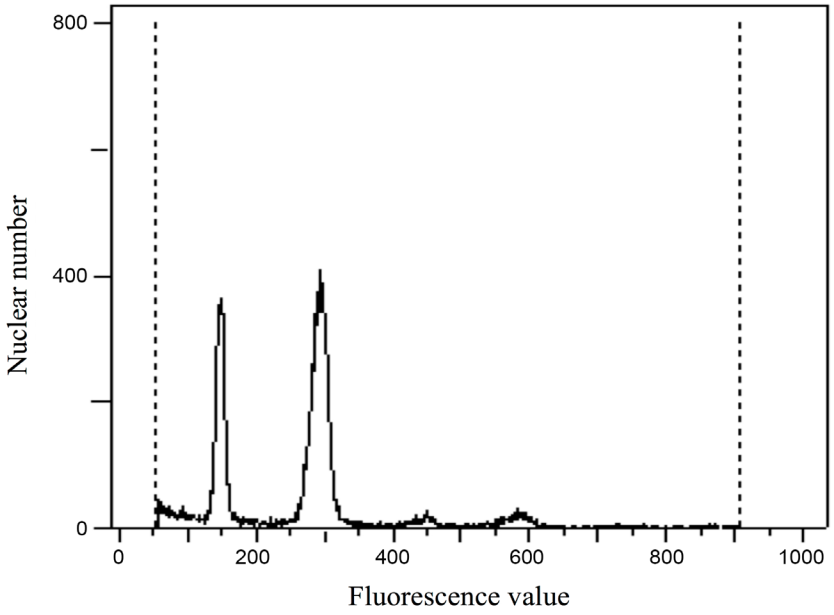


Figure 6. G1 peaks of haploid and disomic lines of *N. tabacum* L. "Red Russian". The DNA content of 20,000 - 22,000 nuclei was determined by flow cytometry.

3.5. qPCR Analysis of CLLs

To select CLLs, qPCR was carried out for 103 BC1 plants screened after flow cytometric analysis; 15 sets of specific SSR markers for 15 linkage groups belonging to the S or S/T subgenome were used in each qPCR analysis as target markers. In the experiments between Haplo-Q and disomic *N. tabacum*, the ΔCt of Haplo-Q plants was taken as a value between 1.23 and 1.57, and ΔCt of disomic plants as a value between 1.83 and 3.17. Based on this information, we interpreted a plant with a ΔCt of a specific SSR marker less than 1.5 as lacking the linkage group on which the specific SSR marker is located; in contrast, a plant with a ΔCt of the specific SSR marker more than 1.5 indicates a disomic. According to this criterion, seven BC1 plants were selected from 103 BC1 plants as candidate CLLs by qPCR (**Figure 8**).

The ploidy of these 7 BC1 plants was confirmed by chromosome analysis. The results for 6 BC1 plants by qPCR corresponded to the resultsof chromosome analysis, and these plants were determined as CLLs (**Table 4** and **Figure 9**). These CLLs lacked 2 - 6 chromosomes, and had different morphology (data not shown), such as stem length, floral pattern and leaf size. Among these CLLs, none lacking linkage group 5 or 10 were obtained; the only CLL lacking linkage group 3 was No. 283, and the only CLL lacking linkage group 6 was No. 184. These CLLs were male sterile, so they must be maintained by a backcross with *N. tabacum* as Haplo-Q lines.

One BC1 plant (No. 332), based on qPCR analysis, was a BC1 plant with 13 linkage groups lacking, but based on chromosome analysis, it was a BC1 plant with 41 chromosomes. It was difficult to tell which chromosome is lacking in this BC1 plant, so this BC1 plant was not used as a CLL.

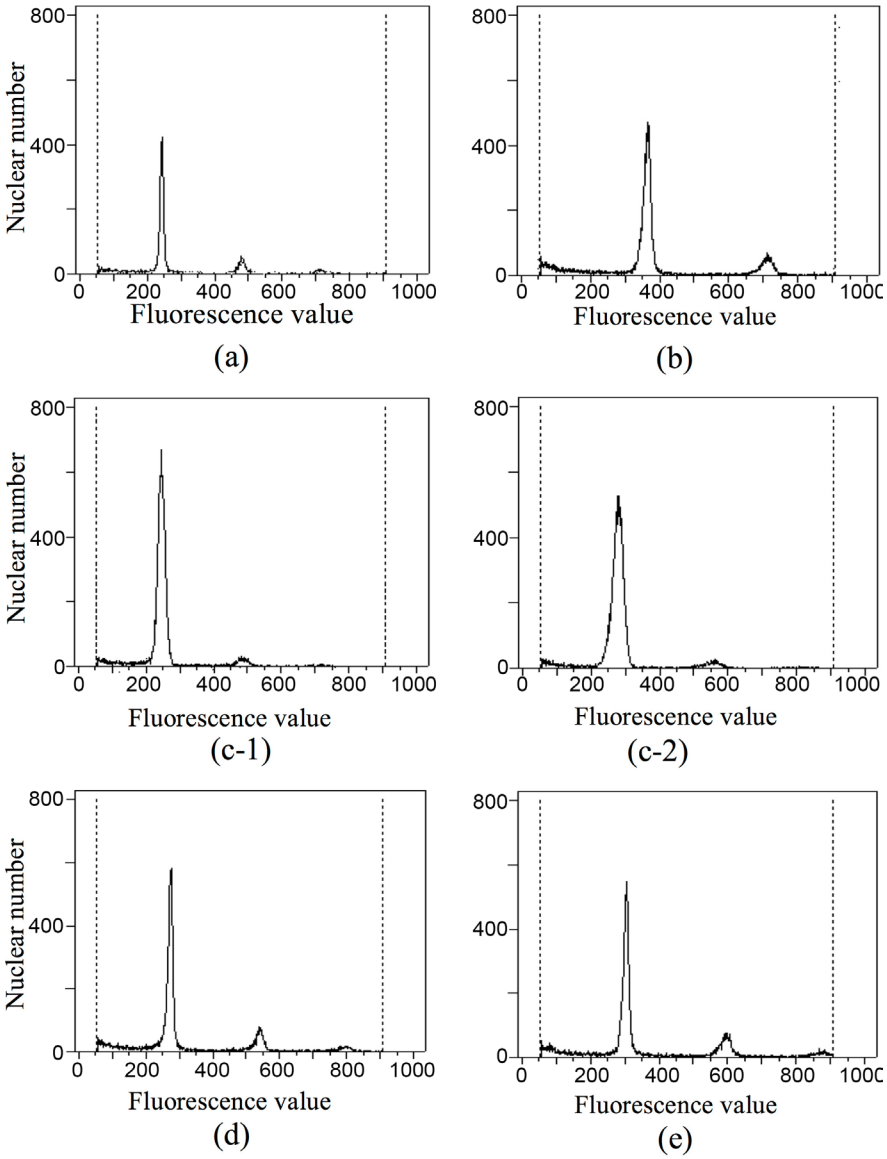
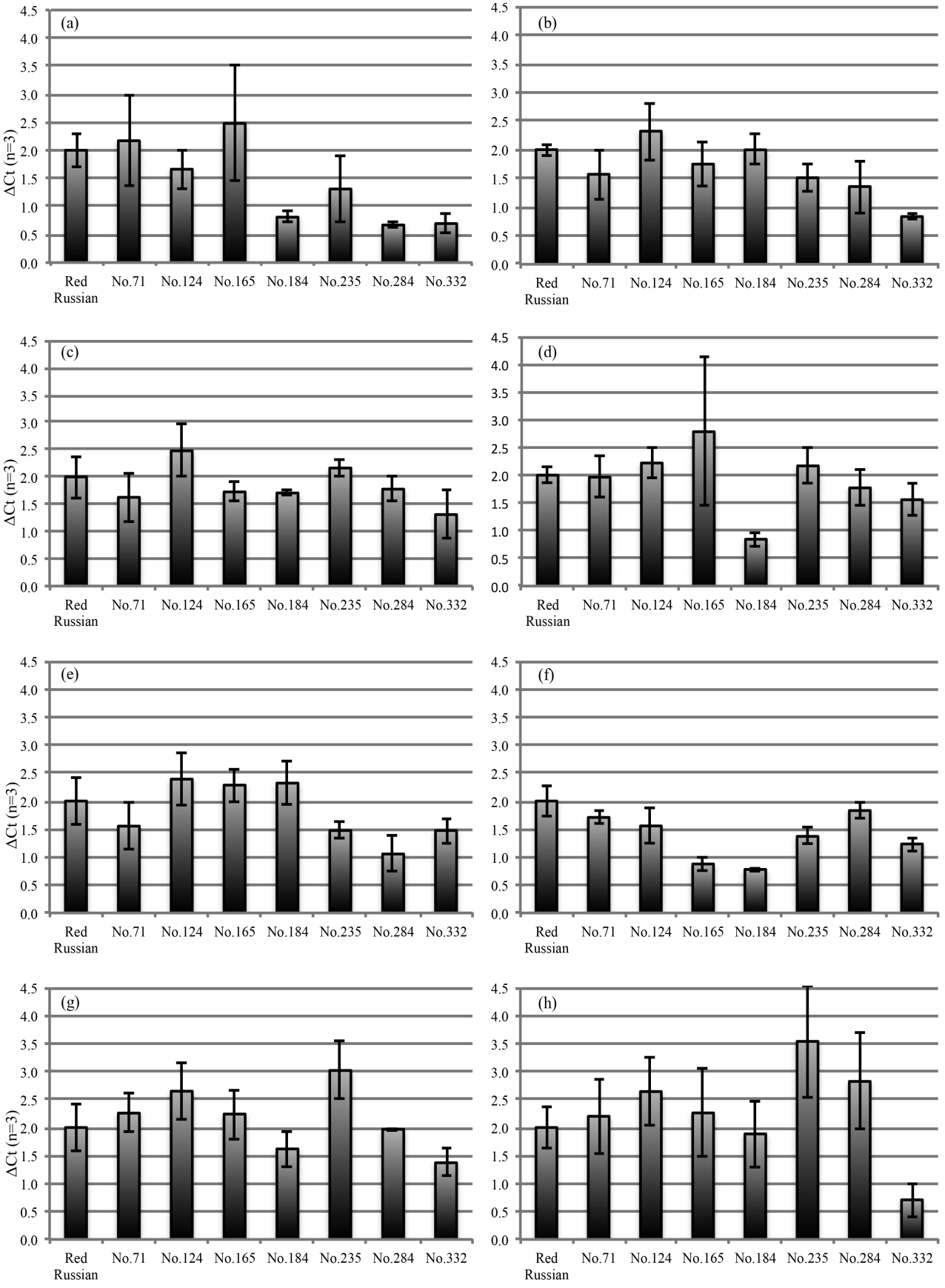


Figure 7. G1 peaks of BC1 by flow cytometry. The DNA content of 20,000 - 22,000 nuclei was determined. (a) The G1 peak of a seedling with DAPI fluorescence values of less than 250; (b) the G1 peak of a seedling with DAPI fluorescence values of more than 300; (c-1, 2) two samples of leaf cells from different leaves of one chimeric BC1 plant; (d) the G1 peak of a seedling with DAPI fluorescence values between 250 and 300; (e) the G1 peak of a seedling with a DAPI fluorescence value of 300.

4. Discussion

Clausen and Cameron [13] reported that the progeny obtained from the cross of (*N. tabacum* × *N. tomentosa*) × *N. tabacum* consist of plants of marked diversity as a consequence of random distribution of 36 - 48 unassociated chromosomes; the offspring consist of unbalanced diploids, which are simple monosomic lines or chromosome aberration lines. They used chromosome analysis and morphological characterisation to detect monosomic lines of *N. tabacum* from the plants with 36 - 48 chromosomes (**Figure 10(a)**). In recent studies, it was determined


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
ΔCt (n=3)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Red Russian
No.71
No.124
No.165
No.184
No.235
No.284
No.332

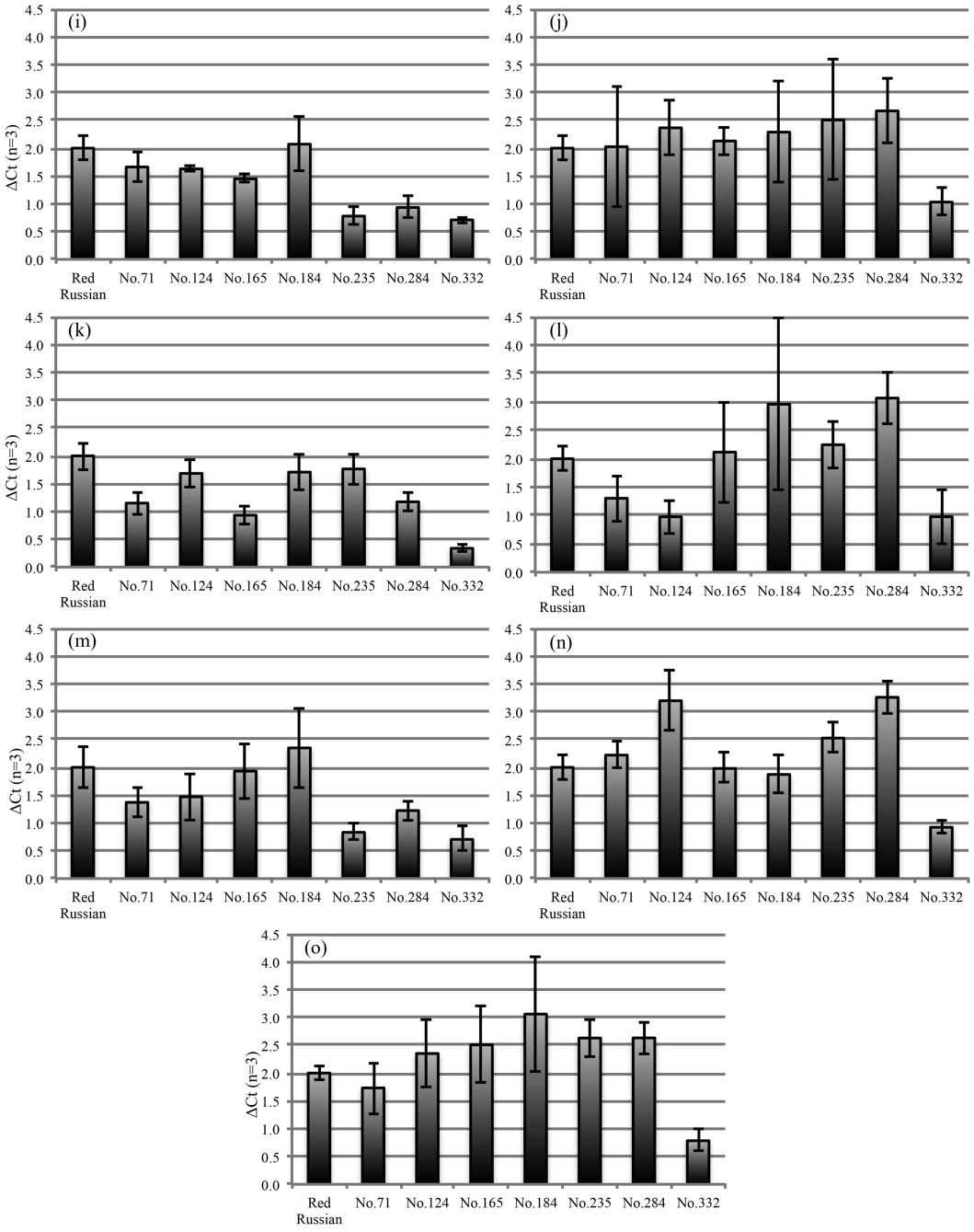
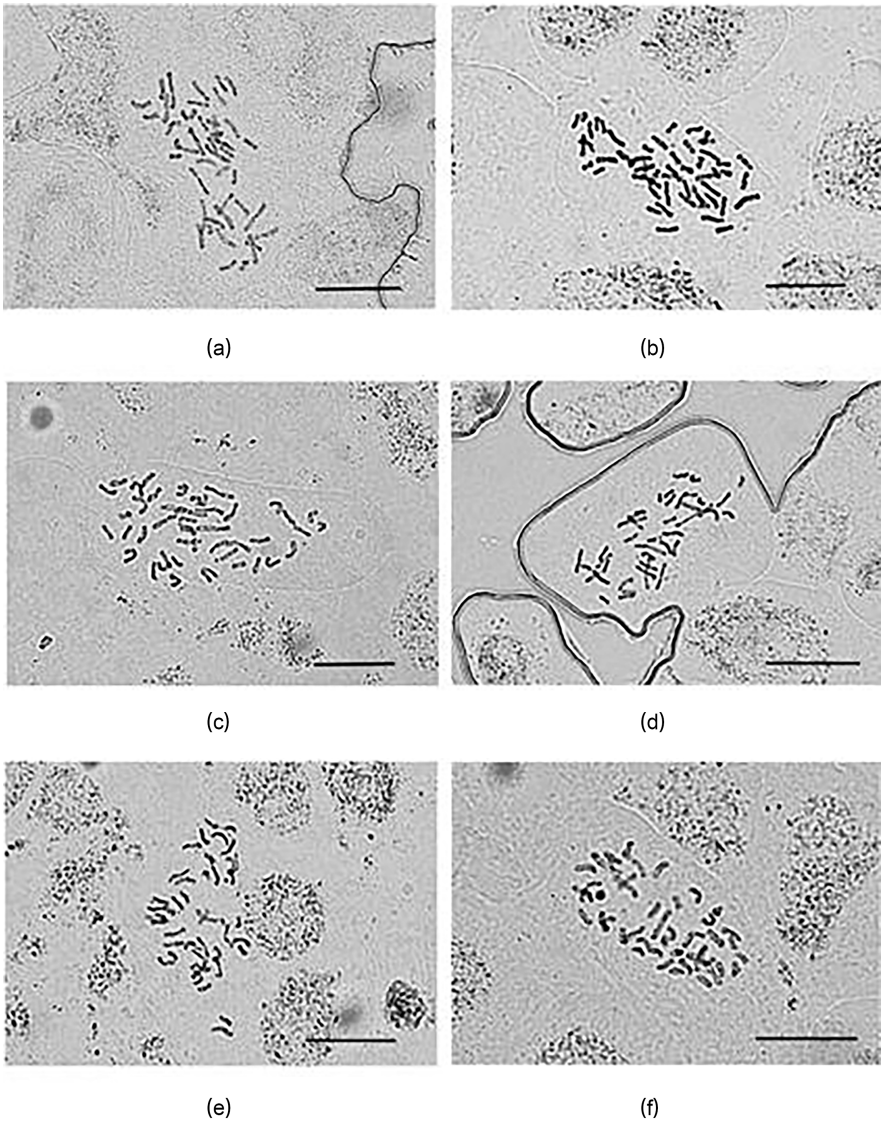


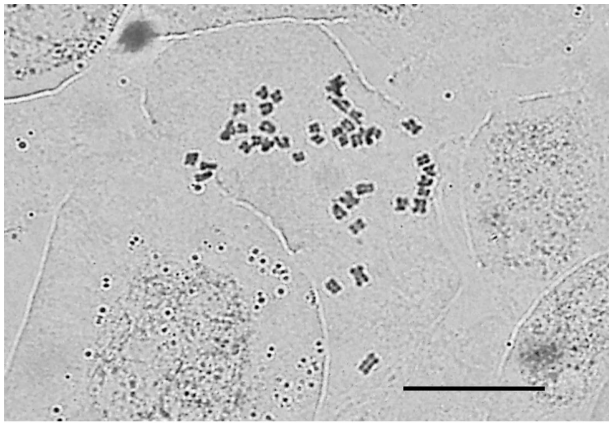
Figure 8. ΔCt of 15 set SSR markers in chromosome lacking lines and*N. tabacum* L. "Red Russian". These 15 sets SSR marker map to chromosomes belonging to the S or S/T genome. (a) ΔCt (Ct Actin - Ct PT30157) values for SSR marker mapping to linkage group 1; (b) ΔCt (Ct Actin - Ct PT30025) values for SSR marker mapping to linkage group 3; (c) ΔCt (Ct Actin - Ct PT30111) values for SSR marker mapping to linkage group 5; (d) ΔCt (Ct Actin - Ct PT30368) values for SSR marker mapping to linkage group 6; (e) ΔCt (Ct Actin - Ct PT30411) values for SSR marker mapping to linkage group 7; (f) ΔCt (Ct Actin - Ct PT30307) values for SSR marker mapping to linkage group 8; (g) ΔCt (Ct Actin - Ct PT30235) values for SSR marker mapping to linkage group 9; (h) ΔCt (Ct Actin - Ct PT30241) values for SSR marker mapping to linkage group 10; (i) ΔCt (Ct Actin - Ct PT30342) values for SSR marker mapping to linkage group 11; (j) ΔCt (Ct Actin - Ct PT1313) values for SSR marker mapping to linkage group 13; (k) ΔCt (Ct Actin - Ct PT30302) values for SSR marker mapping to linkage group 16; (l) ΔCt (Ct Actin - Ct PT30097) values for SSR marker mapping to linkage group 18; (m) ΔCt (Ct Actin - Ct PT40010) values for SSR marker mapping to linkage group 20; (n) ΔCt (Ct Actin - Ct PT30186) values for SSR marker mapping to linkage group 21; (o) ΔCt (Ct Actin - Ct PT30324) values for SSR marker mapping to linkage group 22.

Table 4. List of 6 CLLs.

	LG * No.														
	1	3	5	6	7	8	9	10	11	13	16	18	20	21	22
BC1 No. 71											—**	—	—		
BC1 No. 124												—	—		
BC1 No. 165						—			—		—				
BC1 No. 184	—			—		—									
BC1 No. 235	—				—	—			—				—		
BC1 No. 284	—	—			—				—		—		—		

*LG: linkage group; **—: lacking the linkage group.





(g)

Figure 9. Analysis of chromosomes in chromosome lacking lines. (a) Number of chromosomes in No. 71 (2n = 45); (b) number of chromosomes in No. 124 (2n = 46); (c) number of chromosomes in No. 165 (2n = 45); (d) number of chromosomes in No. 184 (2n = 45); (e) number of chromosomes in No. 235 (2n = 43); (f) number of chromosomes in No. 284 (2n = 42); (g) number of chromosomes in No. 332 (2n = 41). Scale bars: 20 μm.

that one of the paternal progenitors of *N. tabacum* is *N. tomentosiformis*, so here we used *N. tomentosiformis* to cross with *N. tabacum*. In the method of Clausen and Cameron [13], before chromosome analysis, morphological characterisation was used in initial screening to select candidate monosomic lines. However, in experiments for detection of Haplo-Q, nine plants were classified as Haplo-Q plants by qPCR and chromosome analysis, but based on analysis of the percentage of fertile pollen, they were classified asdisomics (**Figure 3** and **Table 3**). This result shows that a method based on morphological characterisation may be misleading. Moreover, morphological characterisation is unstable to changes in the environment. In contrast to screening by morphological characterisation, we used flow cytometric and qPCR analysis to select the CLLs, leading to more objective and exact results that are unaffected by the environment or artificial factors (**Figure 10(b)**).

Clausen and Cameron [13] produced 24 monosomic lines of *N. tabacum*, and these monosomic lines are useful to find the location of one factor on a chromosome that controls a variety of genetic problems in breeding. However, for the determination of more than one factor, we were unable to do more using the monosomic lines, so we also selected CLLs ($40 < 2n \leq 47$).

Unfortunately, we could not get monosomic lines of the S or S/T subgenome of *N. tabacum* from BC1 plants. We decided to select monosomic lines of the S or S/T subgenome of *N. tabacum* from crosses between CLLs and *N. tabacum* using qPCR analysis.

In the present study, comparing Haplo-Q and a disomic, reference ranges for one Q chromosome as represented by a value of ΔCt between 1.23 and 1.57, and for two Q chromosomes, a value of ΔCt between 1.83 and 3.17, were established. The range of the values of ΔCt for Haplo-Q is not linked with values for a

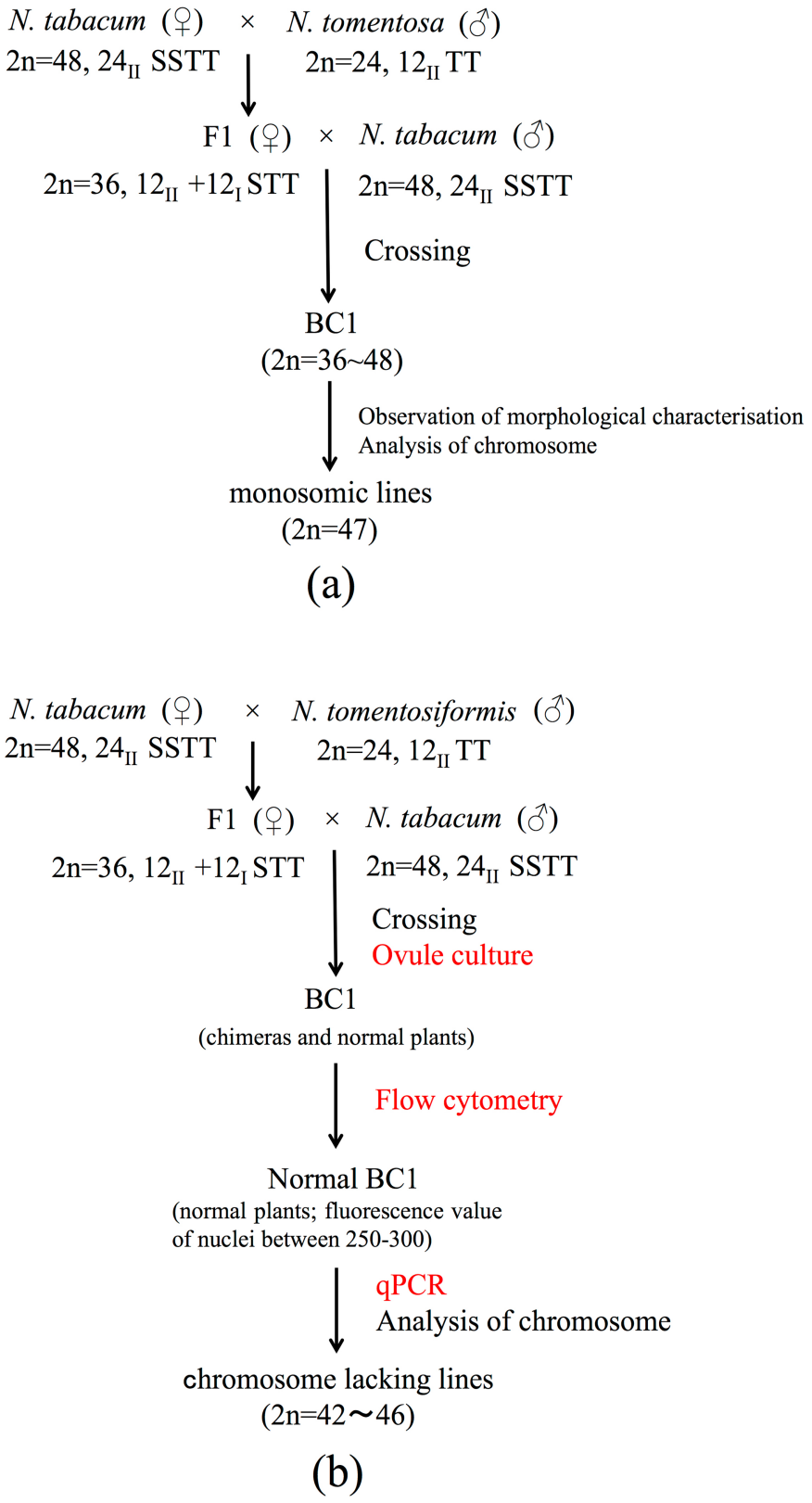


Figure 10. Crossing scheme for production of chromosome lacking lines. (a) The conventional method of Clausen and Cameron [13]; (b) the method developed in this publication.

disomic, but there is no risk of misinterpretation. The data obtained from the SSR marker PT30342 were normalised in relation to Actin, and were calibrated to equivalent data derived from a disomic reference. This method of evaluation partially compensates for discrepancies in DNA quality and deviation in measurements of the DNA concentration, provided there is equal efficiency in amplification of Actin and the SSR marker.

In humans, qPCR has been used as a diagnostic method for detection of trisomy 21 and Klinefelter syndrome, which is performed on a housekeeping gene and the target gene on chromosome fragments, with the difference in the threshold cycle (Ct) of the two genes (ΔCt) calculated to identify an affected or normal patient; based on this method, foetal trisomy 21 or Klinefelter syndrome is rapidly detectable based on gene dosage analysis from amniocytes [18] [19] [20] [31]. Here, we used an SSR marker to carry out the qPCR analysis. The results of qPCR and chromosome analysis in one BC1 plant (No. 332) were not consistent; based on qPCR, No. 332 had 35 chromosomes, but based on chromosome analysis, it had 41 chromosomes. Notwithstanding that these results show a limitation to the qPCR method using SSR markers to select CLLs, qPCR is undeniably a method that can be used to select CLLs. Standardising the specific SSR marker of each linkage group belonging to the S subgenome or S/T subgenome should enable using these SSR marker to carry outqPCR, which should resolve inconsistencies such as that observed for No. 332, and the analysis would be more robust in the selection of CLLs, such as in the detection of trisomy 21 and Klinefelter syndrome.

In the present study, we developed a method to make CLLs belonging to the S or S/T subgenome of *N. tabacum*, which is the first development of a rapid technique for screening of CLLs. Currently, we are attempting to perform crosses of these CLLs of the S or S/T subgenome of *N. tabacum* and *N. nudicaulis*to determine the chromosome of *N. tabacum* that is responsible for hybrid lethality of *N. nudicaulis* × *N. tabacum* crosses.

Here, we only made CLLs of the S or S/T subgenome of *N. tabacum*. We will use the method established in this study to produce CLLs of the T subgenome of *N. tabacum*, and to gather all CLLs of *N. tabacum*. These CLLs would be useful for future analytical studies, such as specific chromosome assignment of genes leading to Mendelian characters including hybrid lethality in the hybrids resulting from crosses between wild species and cultivated tobacco, *N. tabacum*.

5. Conclusion

This was the first successful development of a rapid technique for production of CLLs in *N. tabacum*. The aim of this study was to develop and test a quick and simple screening method for other monosomic lines or chromosome aberrations in *N. tabacum*. Moreover, related qPCR analysis would be helpful in screening other plants having an aberrant number of chromosomes based on an evaluation of ΔCt (Ct housekeeping gene - Ct target gene) of the target genes or markers

that map to a particular chromosome.

Acknowledgements

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