Short Communication

Development of Q-chromosome-specific DNA Markers in Tobacco and Their Use for Identification of a Tobacco Monosomic Line

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We developed seven Q-chromosome-specific DNA markers in Nicotiana tabacum by random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) analysis using two hybrid lines, and we were able to identify tobacco monosomic plants among F1 progeny derived from the cross N. tabacum Haplo-Q × N. tabacum cv. Samsun NN using Q-chromosome-specific DNA markers. Based on the results, we discuss the roles of the Q chromosome in embryo sac development and embryogenesis. Here, we propose a new method for identifying DNA markers for a particular chromosome in the genus Nicotiana.

Keywords: Chromosome-specific DNA marker — Embryogenesis — Embryo sac development — Monosomic — PCR — Tobacco.

Abbreviations: CTAB, cetyltrimethylammonium bromide; DAPI, 4′,6-diamino-2-phenylindole dihydrochloride; FISH, fluorescence in situ hybridization; GISH, genomic in situ hybridization; ISSR, inter-simple sequence repeat; PCD, programmed cell death; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SCAR, sequence characterized amplified region; STS, sequence tagged site.

Nicotiana tabacum (2n = 4x = 48, SSTM) is an amphidiploid originated by interspecific hybridization and subsequent chromosome doubling. From a number of studies, Nicotiana sylvestris (2n = 2x = 24, SS) and Nicotiana tomentosiformis (2n = 2x = 24, TT) are considered to be the progenitors of N. tabacum (Sheen 1972, Gray et al. 1974, Bland et al. 1985). Each chromosome of N. tabacum is lettered in alphabetical order (A–Z, excluding X and Y) in which chromosomes A–L belong to the T subgenome and those of M–Z to the S subgenome. A complete set of 24 monosomic lines of N. tabacum (Haplo-A–Z) have been established on a genetic background of tobacco cultivar Red Russian (var. Purpurea in earlier studies) and they are classified based on morphological characteristics (Clausen and Cameron 1944, Cameron 1959). The monosomic lines are useful for locating genes on specific chromosomes (Clausen and Cameron 1944).

In the genus Nicotiana, the number of chromosomes is large and all chromosomes are relatively small. Therefore, although the karyotype of N. tabacum was described by Goodspeed (1954), discrimination of each chromosome of N. tabacum by karyotype analysis is very difficult. On that account, chromosomes of N. tabacum have been characterized by molecular cytogenetics. Kenton et al. (1993) distinguished the chromosomes into the S and T subgenomes by genomic in situ hybridization (GISH), and demonstrated that some chromosomes were characterized by rDNA loci and intergenomic translocations. Moscone et al. (1996) reported that 20–21 out of 24 chromosome pairs of N. tabacum could be distinguished by simultaneously performing fluorescence in situ hybridization (FISH) and GISH together with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI) counterstaining. Additionally, quantitative idiograms of the T subgenome were constructed based on the measurement of DAPI intensities on chromosomes at the prometaphase stage using a chromosome image analyzing system, CHIAS III; rDNA loci were mapped on the idiograms they developed (Kitamura et al. 2000).

Although some studies to distinguish chromosomes of N. tabacum were reported as mentioned above, DNA markers for each chromosome have not been reported yet. In the present study, we identified PCR-based DNA markers specific to the Q chromosome of N. tabacum. Additionally, we identified a monosomic line with Q-chromosome-specific DNA markers. Based on the results, we discussed the roles of the Q chromosome in embryo sac development and embryogenesis in N. tabacum.

Hybrid seedlings from the cross N. tabacum × Nicotiana suaveolens were obtained by test-tube pollination and ovule culture. However, they expressed hybrid lethality accompanied by programmed cell death (PCD) and died at the cotyledon stage at 28°C (Tezuka and Marubashi 2004). This lethality was suppressed at a temperature of 36°C; therefore, viable hybrid plants (2n = 40) from the cross N. tabacum cv. Red Russian × N. suaveolens could be obtained by cultivation at 36°C (Tezuka and Marubashi 2004).

The genetics of hybrid lethality have been studied in the progeny after the cross between N. tabacum and N. suaveolens. Inoue et al. (1996) used two progenitors of N. tabacum for the cross with N. suaveolens. They reported that the S subge-
Q-chromosome-specific DNA markers in tobacco

Fig. 1 Detection of Q-chromosome-specific DNA markers by RAPD (A–E) and ISSR analysis (F, G). PCR amplifications were performed using primers OPA-06 (A), OPB-07 (B), OPB-13 (C), OPC-11 (D), OPG-04 (E), SP8–1, (TGTC)$_4$ (F) and SP16–1, (CGAA)$_4$ (G). Arrows indicate bands specific to the Q chromosome of *N. tabacum*. M, DNA markers (λ/HindIII and φX174/HaeIII). Lane 1, *N. tabacum* cv. Red Russian; lanes 2, 3, *N. tabacum* cv. Red Russian × *N. suaveolens* cultivated at 36°C; lane 4, Q-1; lane 5, Q-2; lane 6, *N. suaveolens*. 

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Q-chromosome-specific DNA markers in tobacco

Table 1  Nucleotide sequences of 22-mer primers for a STS derived from the RAPD markers

| RAPD marker | Primer | Sequence |
|-------------|--------|----------|
| OPC-11$_{922}$ | QC11–1 | AAAAGCTGCGG GGGCCCTCAT A AAG |
| QC11–2 | OPG-04 –1 | AGCGTGTCGTCGTA ACT GTT TT |
| QC11–2 | OPG-04 –2 | AGCGTGTCGTCGAT AGAAC AATC |

The underlined sequences represent the sequence of the original random primers.

After performing RAPD and ISSR analysis, five and two primers, respectively, generated DNA fragments that were present in the viable hybrid line obtained by cultivation at 36°C and were absent in Q-1 and Q-2 (Fig. 1). These DNA fragments were thought to be specific to the Q chromosome of *N. tabacum*.

We tried converting two Q-chromosome-specific DNA markers generated by primers OPC-11 and OPG-04 to a sequence tagged site (STS) for greater reliability as a selection tool. The amplified DNAs of primers OPC-11 and OPG-04 were resolved by electrophoresis on an 8% polyacrylamide gel in TBE buffer. The bands corresponding to Q-chromosome-specific DNA markers were excised from the gel. They were reamplified with the same primers as those originally employed to generate the bands. The PCR products were resolved by electrophoresis on a 1.5% agarose gel in TBE buffer, and the bands corresponding to Q-chromosome-specific DNA markers were excised from the gel. The DNA fragments were purified using the QIAquick Gel Extraction Kit (Qiagen) and cloned using the pGEM-T Easy Vector System (Promega). Selection of transformed clones was performed by PCR analysis directly on white colonies. The correct clone was confirmed by hybridizing the insert with a Southern blot of RAPD fragments amplified from two hybrid lines. The cloned fragment was sequenced from both ends using T7 and SP6 promoter primers using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

 Sequencing revealed that the DNA fragment generated by the primer OPC-11 was 922 bp. For the DNA fragment generated by the primer OPG-04, the cloned fragment was partly sequenced from both ends. Based on the sequences, 22-mer oligonucleotides were designed as STS primers. Each of these primers contained the original 10 bases of the random primer plus the next 12 internal bases (Table 1). For PCR amplifications with each pair of STS primers, reaction mixtures were the same as those used for RAPD analysis except for 0.2 µM of each primer and 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems). PCR amplifications with QC11–1 and QC11–2 primers were performed in a GeneAmp PCR System 2400 (Applied Biosystems) under the following conditions: 3 min at 94°C for initial denaturation, followed by 35 cycles of 30 s at 94°C, 1 min at 60°C, 1 min at 72°C and a final extension of 5 min at 72°C. PCR amplifications with QG04–1 and QG04–2

nom in *N. tabacum* was responsible for hybrid lethality since hybrid lethality was observed in seedlings from reciprocal crosses between *N. suaveolens* and *N. sylvestris*, whereas hybrid lethality was not observed in seedlings from the cross between *N. suaveolens* and *N. tomentosiformis*. Furthermore, Marubashi and Onosato (2002) carried out a cross between *N. tabacum* monosomic lines of the S subgenome and *N. suaveolens*, and reported that viable hybrid plants (2n = 39) were obtained by cultivation at 28°C when *N. tabacum* Haplo-Q was used as the female parent. Therefore, they concluded that the Q chromosome of *N. tabacum* was responsible for hybrid lethality observed in the cross between *N. tabacum* and *N. suaveolens*.

As mentioned above, we possessed two hybrid lines; i.e. one viable hybrid line (2n = 40) obtained from the cross *N. tabacum* cv. Red Russian × *N. suaveolens* cv. Red Russian × *N. sylvestris* by cultivation at 36°C (Tezuka and Marubashi 2004) and the other viable hybrid line (2n = 39) obtained from the cross *N. tabacum* Haplo-Q × *N. suaveolens* by cultivation at 28°C (Marubashi and Onosato 2002). Two viable hybrid plants of the latter line were considered to lack the Q chromosome of *N. tabacum* and were designated Q-1 and Q-2 (Marubashi and Onosato 2002). To identify Q-chromosome-specific DNA markers, we performed random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) analysis using two hybrid plant lines.

Total DNA was extracted from fresh leaves of *N. tabacum* cv. Red Russian × *N. suaveolens* in the cytohylmethylammonium bromide (CTAB) method (Murray and Thompson 1980) with some modifications. RAPD and ISSR analysis were carried out as described by Rostiana et al. (1999) with some modifications. For RAPD analysis, 200 random primers of 10-mer oligonucleotides (Kit A–J) were used as the female parent. Therefore, they concluded that the Q chromosome of *N. tabacum* was responsible for hybrid lethality observed in the cross between *N. tabacum* and *N. suaveolens*.

For ISSR analysis, 49 non-anchored SSR primers of 16-mer oligonucleotides were used. The original 10 bases of the random primer were replaced with the next 12 internal bases (Table 1). For PCR amplifications with each pair of STS primers, reaction mixtures were the same as those used for RAPD analysis except for 0.2 µM of each primer and 1.5 mM MgCl$_2$, 10 mM Tris-HCl, pH 9.0, 0.2 mM of each dNTP, 2 min at 72°C and a final extension of 5 min at 72°C. For ISSR analysis, 49 non-anchored SSR primers of 16-mer oligonucleotides with four-base repeats (Yang et al. 2001) were amplified. The amplified DNAs of primers OPC-11 and OPG-04 were resolved by electrophoresis on a 1.5% agarose gel in TBE buffer. The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen) and cloned using the pGEM-T Easy Vector System (Promega). Selection of transformed clones was performed by PCR analysis directly on white colonies. The correct clone was confirmed by hybridizing the insert with a Southern blot of RAPD fragments amplified from two hybrid lines. The cloned fragment was sequenced from both ends using T7 and SP6 promoter primers using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

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primers were the same as those described above except for an extension time of 2 min. Optimal amplifications for each marker were determined by varying annealing temperature. In the case of the marker OPC-11922 generated by STS primers, a single band of the same size as fragments generated by primer OPC-11 was detected in *N. tabacum* cv. Red Russian and the viable hybrid line obtained by cultivation at 36°C, but was not detected in *N. suaveolens* (Fig. 2A). In the case of the marker OPG-041700 generated by STS primers, a band of the same size as fragments generated by primer OPG-04 was detected in *N. tabacum* cv. Red Russian and the viable hybrid line obtained by cultivation at 36°C, and another band was detected in all plants except *N. suaveolens* (Fig. 2B). Only the former band corresponding to OPG-041700 was amplified when the annealing temperature was increased to 65°C (data not shown). Therefore, we could successfully convert two Q-chromosome-specific DNA markers generated by primers OPC-11 and OPG-04 to an STS.

We determined whether seven Q-chromosome-specific DNA markers obtained by RAPD and ISSR analysis were detected or not among nine cultivars of *N. tabacum* other than the cultivar Red Russian. Tobacco cultivars used in this study were Hicks-2, Bright Yellow 4, White Burley, Burley 21, Samsun, Samsun NN, Xanthi, Xanthi NN and Xanthi NC. When seven Q-chromosome-specific DNA markers obtained by RAPD and ISSR analysis were tested, the marker OPC-11922 was not detected in cultivars Samsun NN and Xanthi (Fig. 3A). This indicated that OPC-11922 could be used to differentiate the Q chromosome of the cultivar Red Russian from that of cultivars Samsun NN and Xanthi. When PCR amplifications with QC11–1 and QC11–2 primers were performed, OPC-11922 was not detected in the cultivar Samsun NN, but was in the cultivar Xanthi (Fig. 3B). There are several reports that polymorphisms could not be detected by converting RAPD markers to STS or sequence characterized amplified region (SCAR) markers (Paran and Michelmore 1993, Adam-Blondon et al. 1994, Hu et al. 1997). In this regard, Paran and Michelmore (1993) presumed that original RAPD polymorphisms were caused by mismatches in one or a few nucleotides in the priming sites and these mismatches were tolerated by the longer primers.

Since the marker OPC-11922 was detected on the Q chromosome of *N. tabacum* Haplo-Q (the genetic background is the cultivar Red Russian) but not detected on the Q chromosome of the cultivar Samsun NN, we predicted that among F1 progeny derived from the cross between Haplo-Q and the cultivar Samsun NN, F1 hybrid seedlings with OPC-11922 would be disomics and those without OPC-11922 would be monosomics (Fig. 4). Using this approach, we investigated the roles of the Q chromosome in embryo sac development and embryogenesis.

For the cross, Haplo-Q was used as the maternal parent because pollen of Haplo-Q aborts at a high frequency (Cameron 1959). After flowers of Haplo-Q were pollinated with pollen of the cultivar Samsun NN, fertilization and seed set occurred normally. Two hundred and twenty seeds were sown on 1/2 MS medium supplemented with 1% sucrose and 0.2% Gelrite (pH 5.8), and were cultured under continuous illumination (approximately 3,000 lux) for germination. Seed germination was normal and a total of 193 hybrid seedlings were obtained. These seedlings were potted and cultivated under greenhouse conditions. Plant growth and seed germination were normal.

To discriminate between disomics and monosomics in F1 hybrid seedlings from the cross Haplo-Q × Samsun NN, PCR amplifications with QC11–1 and QC11–2 primers were performed (Fig. 5). Among the hybrid seedlings, OPC-11922 was detected in 91 seedlings and was not detected in 102 seedlings. On the other hand, when PCR amplifications with QG04–1 and QG04–2 primers were performed, QG04–1700, which was detected in both Red Russian and Samsun NN cultivars, was detected in all hybrid seedlings (data not shown). This indicated that one Q chromosome from Samsun NN was present in all hybrid seedlings. As mentioned above, although we supposed that hybrid seedlings with OPC-11922 were disomics and hybrid seedlings without OPC-11922 were monosomics, there

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**Fig. 2** PCR amplifications with QC11–1 and QC11–2 primers (A) and QG04–1 and QG04–2 primers (B). M, DNA markers (*λ/HindIII* and *φX174/HaeIII*). Lane 1, *N. tabacum* cv. Red Russian; lanes 2, 3, *N. tabacum* cv. Red Russian × *N. suaveolens* cultivated at 36°C; lane 4, Q-1; lane 5, Q-2; lane 6, *N. suaveolens*.
was a possibility that hybrid seedlings without OPC-11922 were disomics because of recombination. Therefore, 10 hybrid seedlings with OPC-11922 and 10 hybrid seedlings without OPC-11922 were selected at random, and the number of chromosomes in more than five root tip cells for each hybrid seedling was counted (Fig. 6). Chromosome analysis confirmed that the 10 hybrid seedlings with OPC-11922 were disomics and the 10 hybrid seedlings without OPC-11922 were monosomics. These results indicate that a tobacco monosomic line can be identified in F1 progeny derived from the cross between Haplo-Q and the cultivar Samsun NN by Q-chromosome-specific DNA markers. This method would facilitate the rapid identification of a tobacco monosomic line, especially for the Q chromosome, without chromosome analysis.

Monosomic and trisomic lines have been used to locate restriction fragment length polymorphism (RFLP) markers to a specific chromosome in several plant species by the presence or absence of a certain fragment or by its signal intensity (Helentjaris et al. 1986, Young et al. 1987). Use of such RFLP markers would facilitate identification of monosomic or trisomic lines. If RFLP markers specific to the Q chromosome of *N. tabacum* were available, it might be possible to identify Haplo-Q, which is maintained by recurrent backcrossing to the cultivar Red Russian (monosomic × disomic). In general, though, RFLP analysis is laborious and time-consuming, while PCR-based techniques are simple and fast, and allow easy analysis...
of a large number of samples. In the present study, a tobacco monosomic line was identified using PCR-based DNA markers specific to the Q chromosome.

In the process of embryo sac development in tobacco, a megaspore mother cell undergoes meiosis and four megaspores are produced. Then, three megaspores degenerate and one megaspore develops into an embryo sac (Lobanova and Enal'eeva 1998). In the present study, monosomics were obtained at the same rate as disomics from the cross Haplo-Q × Samsun NN. Since \textit{N. tabacum} is an amphidiploid, the T subgenome may have compensated for the lack of the Q chromosome. Nevertheless, if the Q chromosome played crucial roles in embryo sac development, lack of the Q chromosome would have a serious impact on embryo sac development and it would be expected that either monosomics would be obtained at a low rate or no monosomics would be obtained. Therefore, these results suggest that the Q chromosome of \textit{N. tabacum} is not involved in embryo sac development. Furthermore, the germination rate was normal, and abnormal growth was not observed in hybrid seedlings from the cross Haplo-Q × Samsun NN. These observations indicate that the absence of one Q chromosome of \textit{N. tabacum} has no effect on embryogenesis. Thus, we concluded that embryo sac development of Haplo-Q and embryogenesis of monosomic progeny derived from Haplo-Q are normal.

Hybrid seedlings from the cross \textit{N. tabacum} × \textit{N. suaveolens} expressed hybrid lethality accompanied by PCD at 28°C (Tezuka and Marubashi 2004). Since, in this cross combination, viable hybrid plants \((2n = 39)\) were obtained at 28°C when Haplo-Q was used as the female parent, it was thought that the Q chromosome of \textit{N. tabacum} was responsible for hybrid lethality (Marubashi and Onosato 2002). In these experiments, although 23 hybrids were obtained, only four hybrids were viable. Considering the results in the present study that monosomics and disomics were obtained equally from the cross Haplo-Q × Samsun NN, the number of viable hybrids from the cross Haplo-Q × \textit{N. suaveolens} might be too small. We are now trying to clarify why the number of viable hybrids is less than that of lethal hybrids.

In the present study, we could identify Q-chromosome-specific DNA markers using two hybrid lines, i.e. one hybrid line had the Q chromosome and another hybrid line did not. This indicates that chromosome-specific DNA markers in \textit{N. tabacum} could be identified using two hybrid lines obtained from the cross between \textit{N. tabacum} monosomic lines and wild species of the genus \textit{Nicotiana}. Furthermore, we proposed a method for identifying tobacco monosomic lines using chromosome-specific DNA markers and a method for identifying chromosomes involved in embryo sac development and embryogenesis.

**Acknowledgments**

We thank Dr. T. Kuboyama, associate professor of Ibaraki University, for his valuable advice. We are grateful to Dr. T. Kubo, a former director of the Iwata tobacco experiment station of Japan Tobacco Inc., for providing tobacco monosomic lines and to Japan Tobacco Inc. for providing seeds of tobacco cultivars and \textit{N. suaveolens}. This work was partly supported by Grant-in-Aid for Scientific Research (A) No. 13306003 and (C) No. 15580003 from the Ministry of Education, Culture, Sports, Science and Technology, Japan.
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(Received June 28, 2004; Accepted September 13, 2004)