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PCR amplification of CMS-specific mitochondrial nucleotide sequences to identify cytoplasmic genotypes of onion (*Allium cepa* L.)

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Abstract S-cytoplasm is the most common source of cytoplasmic-genic male sterility (CMS) used to produce hybrid onion seed. CMS determinants are thought to be on the mitochondrial (mt) genome. Although the characterization of polymorphisms in the mtDNA that distinguishes the N- and S-cytoplasms is significantly faster than test crossing, it is still time-consuming to complete RFLP analysis. I identified an unusual transcript pattern for the mitochondrial *cob* gene in S-cytoplasm. Sequencing of the mitochondrial *cob* gene revealed an insertion of chloroplast DNA sequence into the upstream region of *cob* in the S-cytoplasm. This region could be amplified with oligonucleotides flanking the upstream region to *cob*. PCR-detectable polymorphism based on differences in the sequences of the mtDNA that distinguishes N- and S-cytoplasms allows a quick and confident identification of the cytoplasm of individual plants.

Key words *Allium cepa* · Mitochondrial DNA · Cytoplasmic male sterility · Polymerase chain reaction

Introduction

The production of hybrid onion seed became economically feasible with the discovery of cytoplasmic-gene male-sterility (CMS) systems (Jones and Emsweller 1936; Berninger 1965). In 1925, Jones and colleagues found a male-sterile plant in a population of the variety 'Italian Red'. Sterility in this plant was conditioned by the interaction of the cytoplasm (S-cytoplasm) with a single nuclear restore gene. In S-cytoplasm, fertility is restored by a dominant allele (*Ms*) at the restorer locus (Jones and Clarke 1943). At present, S-cytoplasm is the most common source of CMS used to produce hybrid-onion seed (Havey 1995).

CMS determinants are thought to be on the mitochondrial genome (Newton 1988). The mitochondrial (mt) DNAs of the male-fertile (normal) and S-cytoplasmic onions have been reported to give distinctive restriction profiles (de Courcel et al. 1989; Holford et al. 1991; Havey 1993; Satoh et al. 1993). The characterization of polymorphisms in the mtDNA that distinguish the N- and S-cytoplasms is a significantly faster procedure than test crossing, but it is still time-consuming to complete a restriction fragment length polymorphism (RFLP) analysis (Havey 1995). The polymerase chain reaction (PCR) would allow a quick and confident identification of the cytoplasm of individual plants. In this report, I present PCR-detectable polymorphism based on the differences in sequences of the mtDNA that distinguishes N- and S-cytoplasms.

Materials and methods

Northern hybridization

In order to find differences in mitochondrial gene expression, I compared the transcriptional properties of mitochondrial genes between W202B (N *msms*) and W202A (S *msms*), and the S-cytoplasmic plant restored to fertility by the nuclear *Ms* allele (S *Msms*). Mitochondria and mtRNA were isolated from the sprouting onion leaves. mtRNA was purified by phenol-chloroform extraction, ethanol precipitation and LiCl precipitation. Purified mtRNA was electrophoresed in 1.17% agarose gel and transferred onto nylon-membrane. Mitochondrial gene probes, *rrn18*, *rrn26*, *coxI*, *coxII*, *cob* and *atpA*, were hybridized to RNA. The clones of *rrn26* and *rrn18* coding for 26S ribosomal RNA and 18S ribosomal RNA, respectively, were from pea (Huh and Gray 1982). The clones of *coxI* and *coxII* coding for cytochrome oxidase subunit 1 and 2, respectively, were isolated from

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sugar beet (Senda et al. 1991). The *cob* coding for apocytochrome B was from wheat (Boer et al. 1985). The clones of *atpA* coding for ATPase subunit alpha were isolated from pea (Morikami and Nakamura 1987). Hybridization, labeling of probe DNA and visualization of the probe-target DNA-RNA hybrid were carried out by the ECL method (Satoh et al. 1993).

Cloning and sequencing of the DNA preparation

Mitochondria and mtDNA were isolated from plants with N-cytoplasm and S-cytoplasm by a combination of differential centrifugation and DNase I treatment (Satoh et al. 1993). Clone libraries of mtDNAs, which were partially digested with *MboI* and size-selected for an insert size of 16–23 kb, were prepared in the phage vector lambda EMBL. The phage clones containing the *cob* gene were isolated by plaque hybridization. DNA fragments with *cob* were subcloned into the pUC 118 and subjected to dideoxy nucleotide sequencing by the auto sequencing (Applied Biosystems).

PCR amplification of cytoplasm-specific nucleotide sequences

Oligonucleotides which flank the nucleotide sequences specific to S- and N-cytoplasms were commercially synthesized and used to amplify mtDNA fragments. Crude mtDNA was isolated from sprouting onion leaves. A 50-mg aliquot of onion leaves was homogenized in buffer (50 m*M* TRIS-HCl pH 7.5, 5 m*M* EDTA, 0.4 *M* sucrose, 0.1 *M* BSA, 5 m*M* 2-mercaptoethanol) and centrifuged at 1,000 rpm for 5 min. The supernatant was then centrifuged at 10,000 rpm for 10 min. The pellet was resolved with lysis buffer (200 m*M* TRIS-HCl pH 9.0, 10 m*M* EDTA,100 m*M* NaCl, 0.5% SDS, 14 m*M* 2-mercaptoethanol, 2% SDS) and placed for 5 min at room temperature. After phenol-chloroform extraction, the DNA was precipitated with ethanol. Crude mtDNA was mixed with 50 m*M* KCl, 10 m*M* TRIS-HCl pH 8.3, 2.5 mM $MgCl_2$, 0.2 m*M* each of dATP, dCTP, dGTP and dTTP, 1.0 unit of *Taq* polymerase (Takara, Kyoto, Japan), and 1 μM of each primer in a 25-μl volume. After an initial period at 94°C for 4 min, 25 cycles were completed at 94°C for 1 min, 52°C for 2 min, 72°C for 3 min. Amplified DNA was electrophoresed in 1.0% agarose gel.

Fig. 1 Northern blot analysis of mtRNA from W202B (*N*, N-cytoplasm), W202A (*S*, S-cytoplasm) and nuclear-restored hybrid with S-cytoplasm (*F*). Respective mtRNA was electrophoresed in 1.17% agarose-formaldehyde gel, transferred onto a nylon filter and hybridized with *rrn26*, *rrn18*, *cob*, *coxI*, *atpA*, or *coxII* probes. Size markers are indicated in kilobases

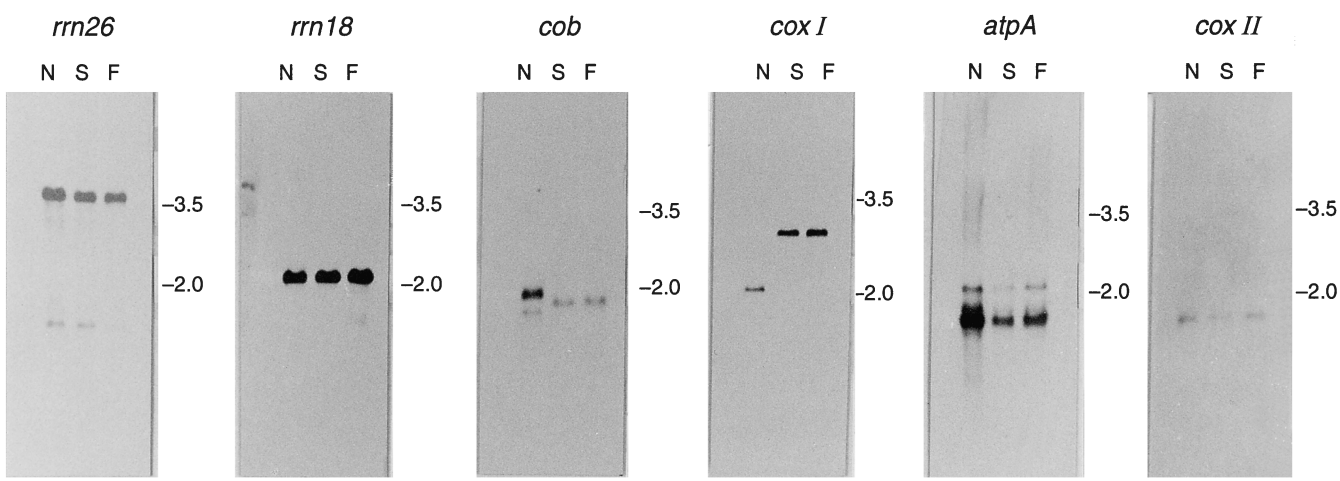
Results and discussion

Northern hybridization

Northern analyses with the *rrn18*, *rrn26*, *coxI*, *coxII*, *cob* and *atpA* probes on mtRNA from plants with N- or S-cytoplasm showed differences in the transcript patterns of *coxI* and *cob* between N- and S-cytoplasms (Fig. 1). For example, 1.85 kb and 1.55 kb of transcripts of *cob* were detected for the N-cytoplasm while 1.70 kb of transcript was detected for the S-cytoplasm. The variation in transcript patterns of *coxI* and *cob* detected among the N- and S-cytoplasms indicates that mitochondrial genome configuration differences exist within these genes or the regulational regions of those genes. No effect of restorer gene to the transcriptional pattern in the S-cytoplasm was found (Fig. 1).

Cloning and sequencing of the *cob* gene

The restriction enzyme maps of the mtDNA fragment containing *cob* from N- and S-cytoplasms indicate that the S-mtDNA diverged from N-mtDNA at the upstream region to *cob* (Fig. 2). Fragments containing the *cob* gene from N- and S-mtDNA were subcloned and used for sequencing. A 1242-bp open reading frame, capable of encoding a polypeptide of 414 amino acids, was identified as *cob* on the basis of DNA sequence homology (92.6% with the corresponding gene of rice). The sequences of the coding region (position 1–1242) and region downstream (position 1243–1741) of S-*cob* were completely homologous to N-*cob* (data not shown); however, the S-*cob* gene diverged from the N-*cob* gene 54 bp 5′ to the initiation codon (Fig. 3). Computer searches of the sequence in the region (position from -53 to -523 to the initiation codon of S-*cob*) detected a significant similarity (94.3%) with tobacco chloroplast *ORF1708* (Fig. 3). This reveals an insertion of chloroplast DNA sequence into the upstream region of S-*cob*, and it contributed to the



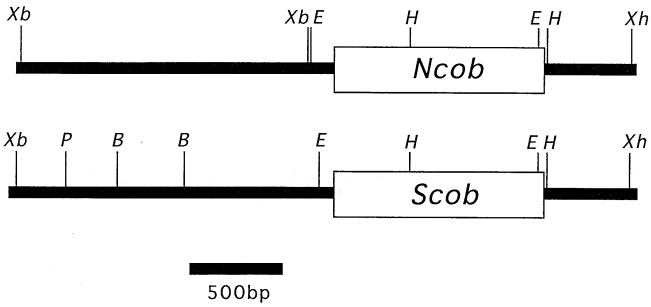


Fig. 2 Restriction map of the region of onion mtDNA containing the *cob* gene. *N-cob*; the *cob* gene from normal cytoplasm, *S-cob* the *cob* gene from sterile cytoplasm, *B*, *Bam*HI, *E Eco*RI, *H Hin*dIII, *P Pst*I, *Xb Xba*I, *Xh*, *Xho*I

rearrangements specific to the S-cytoplasm of onion. A number of chloroplast sequences have been reported in the mitochondrial genomes of higher plants (Stern and Lonsdale 1982). These sequences of chloroplast origin contribute to the exceptionally large size of plant mitochondrial genomes (Schuster and Brennicke 1987). Interorganellar transfer of genetic information is considered to occur via RNA and subsequent local reverse transcription and genomic integration (Schuster and Brennicke 1987).

The rearrangement by the insertion of chloroplast DNA sequence into the 5′ flanking region of S-*cob* accounts for the transcriptional alteration of the *cob* gene in S-cytoplasm. Mitochondrial gene rearrangements and transcriptional alterations are associated with CMS in radish (Makaroff and Palmer 1988), sunflower (Siculella and Palmer 1988) and sugar beet (Senda et al. 1993). The detailed transcriptional and in-organellar translation analysis using normal, CMS and nuclear-restored genotypes could permit a further investigation of the molecular basis of the CMS phenotype in onion.

PCR amplification of cytoplasm-specific nucleotide sequences

Oligonucleotides flanking the upstream region to S- and N-*cob* were commercially synthesized (S-specific primer, 5′-GTCCAGTTCCTATAGAACCTATCACT-3′; N-specific primer, 5′-TCTAGATGTCGCATCAGTGGAATCC-3′; common primer, 5′-CTTTTCTATGGTGACAACTCCTCTT-3′). Relative to the initiation codon of *cob* the S-cytoplasm-specific primer corresponds to position − 418 to − 393; the N-cytoplasm-specific primer to position − 184 to − 160; the common primer to position − 29 to − 5. The primers were used to amplify mitochondrial nucleotide sequences specific to cytoplasmic genotypes. A 414-bp fragment was amplified from the S-cytoplasm and a 180-bp fragment from the N-cytoplasm (Fig. 4). These fragments can be easily distinguished by agarose gel electrophoresis. With these PCR-detectable markers, the cytoplasmic genotypes of individual plants of 'Sapporo-ki' were examined (Table 1). This OP cultivar is considered to be derived from U.S. cultivar 'Yellow Globe Danvers', which was probably introduced to Japan in 1871. This cultivar now exists as several strains. There was variation in the frequency of S-cytoplasm among these strains. For example, 'Yamamoto' had 19 plants with S-cytoplasm and 1 plant with N-cytoplasm. In contrast, 'Hayashi' did not have any plants with S-cytoplasm and 20 plants with N-cytoplasm. This means the probability of extracting maintainer lines from 'Yamamoto' line is much lower than from 'Hayashi'.

Havey (1995) also reported polymorphisms of cytoplasmic genotypes in several OP populations including

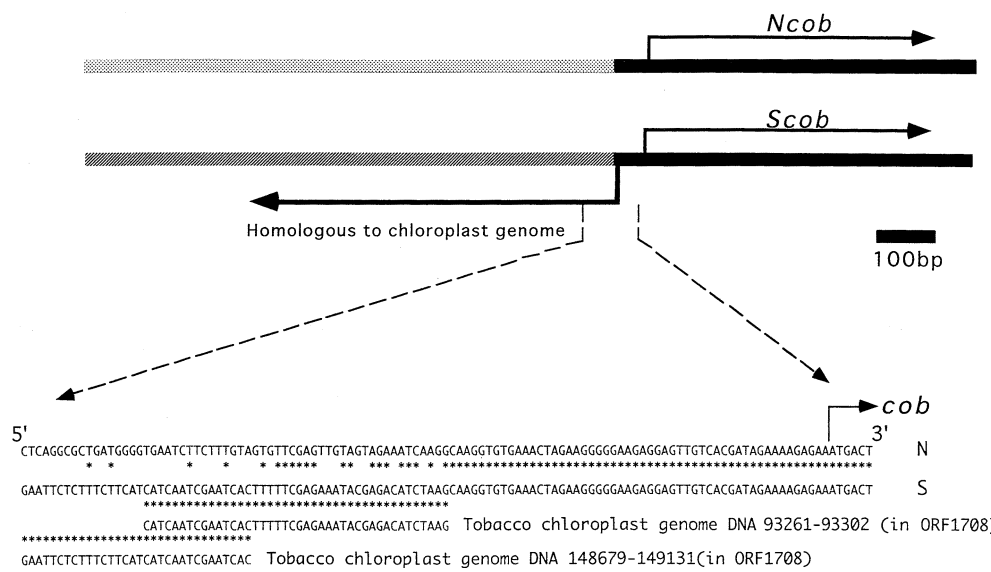


Fig. 3 Nucleotide sequence of upstream region to *cob* from normal cytoplasm and sterile cytoplasm. The sequence of the structural region of the *cob* gene from sterile cytoplasm (S-*cob*) was completely homologous to the *cob* gene from normal cytoplasm (N-*cob*) but diverged from the N-*cob* gene 56 bp 5′ to the initiation codon. This region of S-*cob* was highly homologous to the part of *ORF1708* in tobacco chloroplast DNA. *N* DNA fragment containing *cob* from N-cytoplasm, *S* DNA fragment containing *cob* from S-cytoplasm

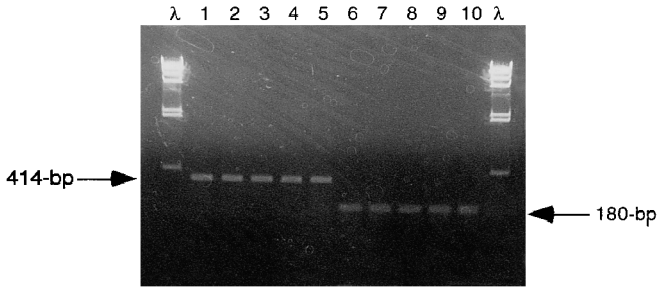


Fig. 4 Amplification of mitochondrial nucleotide sequences specific to normal and sterile cytoplasms from crude mtDNA using primers flanking the upstream region to S- and N-*cob*. *Lane 1* W202A with S-cytoplasm, *lane 2* W420A with S-cytoplasm, *lane 3* 2935A with S-cytoplasm, *lane 4* 611A with S-cytoplasm, *lane 5* K80015A with S-cytoplasm, *lane 6* W202B with N-cytoplasm, *lane 7* W420B with N-cytoplasm, *lane 8* 2935B with N-cytoplasm, *lane 9* 611B with N-cytoplasm, *lane 10* K80015B with N-cytoplasm. *λ*, *λ*DNA digested with *Hin*dIII

Table 1 Frequency of cytoplasms in different strains of open-pollinated onion population 'Sapporo-ki'

Strain	Number of plants with N-cytoplasm	Number of plants with S-cytoplasm
Inoue	13	5
Takahata	10	6
Takada	5	13
Kubo	2	16
Miyamoto	7	12
Uesaki	5	14
Kurokawa	7	10
Takenaka	0	20
Iwanami	8	9
Kamitokoro	13	7
Shimizu	13	6
Kitabayashi	3	15
Yamamoto	19	1
Hayashi	0	20
Chinami	3	7

'Sapporo-ki' using the amplification of a fragment that carries an autapomorphic 100-bp insertion in the chloroplast DNA of the N-cytoplasm and concluded that PCR-detectable markers offered a significantly quicker and cheaper alternative to crossing or Southern analysis.

For a population such as 'Sapporo-ki' in a mixture of N- and S-cytoplasms, the molecular identification of cytoplasms can significantly reduce the number of individual pairings with a sterile tester that are required to identify a maintaining genotype because the plants with S-cytoplasm can be eliminated before testcrossing.

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References

Berninger B (1965) Contribution a l'etude de la sterilite-male de l'oignon (*Allium cepa* L.). Ann Amelior Plant 15:183–199

Boer H, McIntosh J, Gray M, Boen L (1985) The wheat mitochondrial gene for apocytochrome b: absence of a prokaryotic ribosome binding site. Nucleic Acids Res 13:2281–2292

De Courcel AGL, Vedel F, Boussac JM (1989) DNA polymorphism in *Allium cepa* cytoplasms and its implications concerning the origin of onions. Theor Appl Genet 77:793–798

Havey M (1993) A putative donor of S-cytoplasm and its distribution among open-pollinated populations of onion. Theor Appl Genet 81:752–757

Havey M (1995) Identification of cytoplasms using the polymerase chain to aid in extraction of maintainer lines from open-pollinated populations of onion. Theor Appl Genet 90:263–268

Holford P, Croft J, Newbury H (1991) Differences between, and possible origins of, the cytoplasms found in fertile and male-sterile onions (*Allium cepa* L.). Theor Appl Genet 82:737–744

Huh T, Gray M (1982) Conservation of ribosomal RNA gene arrangement in the mitochondrial DNA of angiosperms. Plant Mol Biol 1:245–249

Jones HA, Clark AE (1943) Inheritance of male sterility in the onion and production of hybrid seed. Proc Am Hortic Sci 43:189–194

Jones HA, Emsweller SL (1936) A male-sterile onion. Proc Am Soc Hortic Sci 34:582–585

Makaroff CA, Palmer JD (1988) Mitochondrial DNA rearrangements and transcriptional alterations in the male-sterile cytoplasm of Ogura radish. Mol Cell Biol 8:1474–1490

Morikami A, Nakamura K (1987) Structure and expression of pea mitochondrial F-1 α-subunit gene and its pseudogene involved in homologous recombination. J Biochem 101:967–976

Newton K (1988) Plant mitochondrial genomes: organization, expression and variation. Annu Rev Plant Physiol Plant Mol Biol 39:503–532

Satoh Y, Nagai M, Mikami T, Kinoshita T (1993) The use of mitochondrial DNA polymorphism in the classification of individual onion plants by cytoplasmic genotypes. Theor Appl Genet 86:345–348

Schuster W, Brennicke A (1987) Plastid, nuclear and reverse transcriptase sequences in the mitochondrial genome of *Oenothera*: is genetic information transferred between organelles via RNA? EMBO J 6:2857–2863

Senda M, Harada T, Mikami T, Sugiura M, Kinoshita T (1991) Genomic organization and sequence analysis of the cytochrome oxidase subunit 2 gene from normal and male-sterile mitochondria in sugarbeet. Curr Genet 19:175–181

Senda M, Mikami T, Kinoshita T (1993) The sugar beet mitochondrial gene for the ATPase alpha-subunit: sequence, transcription and rearrangements in cytoplasmic male-sterile plants. Curr Genet 24:164–170

Siculella L, Palmer J (1988) Physical and gene organization of mitochondrial DNA in fertile and male-sterile sunflower. CMS-associated alterations in structure and transcription of the *atpA* gene. Nucleic Acids Res 16:3787–3799

Stern D, Lonsdake D (1982) Mitochondrial and chloroplast genomes of maize have a 12-kilobase DNA sequence in common. Nature 299:698–702