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A TGMS-linked nuclear DNA marker as originated from the mitochondrial genome in rice (*Oryza sativa* L.)

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A nuclear RAPD marker, $OPF18_{2600}$, was linked to a thermosensitive genetic male sterility (TGMS) gene (*tms3*) in a rice mutant line IR32364TGMS. Segregation analysis in two mapping populations and reciprocal cross analysis involving different genotypes indicated, however, that this marker was maternally inherited. The RAPD marker was then cloned as pF18 and partially sequenced from both ends of the insert. Sequence analysis showed that pF18 was homologous to mitochondrial genes, *cob2* and *nad1*. Evidence was provided that the *cob2* DNA segment near pF18 was genetically linked to *tms3*. Thus pF18 could be the result of integration of a piece of mitochondrial DNA segment into the nuclear genome. The transferred DNA segment included at the least *cob2*, a portion of *nad1*, and intergenic sequences. The presence of intron and intergenic sequences in the transferred DNA fragment indicated that DNA was the transfer intermediate. The relationship between the transferred DNA and *tms3* is discussed.

Keywords: evolution, mitochondrial genes, RAPD, rice, sequence homology, TGMS.

Introduction

In Thermosensitive Genetic Male Sterility (TGMS), temperature is the key factor in determining the male fertility/sterility reaction of a genic sterile line. It has been reported that TGMS in rice is inherited in a normal Mendelian fashion and is under the control of a single recessive gene (Yang *et al*., 1990; Maruyama *et al*., 1991). Mapping analysis indicates that one of the TGMS genes is located on rice chromosome 8 (Wang *et al*., 1995). The molecular basis of TGMS is, however, poorly understood at present. On the contrary, cytoplasmic male sterility (CMS) has been widely studied and appears to be associated with DNA rearrangements in a specific mitochondrial region resulting in the synthesis of altered proteins thought to interfere with pollen development (for reviews see Hanson, 1991; Saumitou-Laprade *et al*., 1994; Vedel *et al*., 1994).

Recently, IRRI has developed a TGMS line, IR32364TGMS, which was induced by gamma irradiation (Virmani & Voc, 1991). This gene was also found to be inherited in Mendelian fashion and was controlled by a single recessive gene, *tms3* (Borkakati & Virmani, 1996). We initiated a study on this TGMS line with the final objective to elucidate the molecular basis of TGMS and to apply it to rice hybrid seed production. We identified four RAPD markers linked to *tms3* and located *tms3* on the short arm of chromosome 6 (Subudhi *et al*., 1997). One of the RAPD markers, $OPF18_{2600}$ was very peculiar. It was inherited as a nuclear marker in a Mendelian manner in the *tms3* mapping population, but was maternally inherited in other populations. This poses several intriguing questions. Is the marker nuclear inherited or maternally inherited? If nuclear inherited, why does it show cytoplasmic inheritance? If it is maternally inherited, is the marker locus in the chloroplast or mitochondrial genome? Can this be a piece of DNA from the cytoplasm integrated into the nuclear genome or vice versa? If there is transposition of a DNA fragment between genomes, what is the relationship between transposition and TGMS, if any?

Although we cannot answer all the questions in one study, we provide evidence based on segregation analysis, reciprocal cross analysis and a sequence

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homology search of the DNA sequence database, that $OPF18_{2600}$ is a mitochondrial-genome derived nuclear marker which cross-hybridizes to cytoplasmic DNA in other breeding lines. We hypothesize that this insertion represents a movement of DNA sequence from mitochondrion to nucleus.

Materials and methods

Plant materials and RAPD marker linked to tms3

An F_2 population, from the cross IR32364TGMS (*indica*) × IR68 (*indica*), was used to map *tms3* via bulked segregant analysis (Michelmore *et al*., 1991). The TGMS mutant line, IR32364TGMS, showed complete male-sterility at day/night temperatures of 32°/24°C but partial fertility at 27°/21°C in an IRRI phytotron. A RAPD marker, $OPF18_{2600}$, amplified by the random primer OPF18 (5′-TTCCCGGGTT-3′) was found to be linked to *tms3* (Subudhi *et al*., 1997).

Cloning and sequencing of $OPF18_{2600}$

The $OPF18_{2600}$ was reamplified twice to ensure uniformity of the fragment and purified by using a Geneclean kit (Bio 101). Then the purified fragment was cloned into pMosblue vector using a TA cloning system (Amersham Co.) by direct ligation. The cloned insert was spliced out by double digestion with *Eco*RI and *Xba*I to determine the correct size of the insert which should be 2.6 kb. The identity of the cloned RAPD fragment was verified by hybridization of the cloned fragment to Southern blots of RAPD products amplified with OPF18 primer using DNA from the homozygous sterile and homozygous fertile individuals. The verified clone was named as pF18.

Double-strand sequencing of both ends of the clone was carried out by the dideoxy chain termination method (Sanger *et al*., 1977) using α^{32}P dCTP and the Thermosequenase cycle sequencing kit (Amersham Co.). Based on the sequence information, primers (see Fig. 5 for primer sequences) were designed and another round of sequencing was carried out to obtain more sequence information.

Mapping population and reciprocal crosses

An effort was made to map pF18 onto rice chromosomes through the use of two mapping populations. At first, a doubled haploid population of 135 lines developed by anther culture from the cross between IR64 (*indica*) and Azucena (*japonica*) (Guiderdoni *et al*., 1992) was used, for which an RFLP framework map is available (Huang *et al*., 1994). The second mapping population was an F_2 population derived from the cross of Tesanai 2 × CB, obtained from the China National Rice Research Institute, China (Lin *et al*., 1996). Tesanai 2 is an *indica* variety from Guangdong, China and CB, a variety inclining to *indica* type, is from California, USA. Another F_2 population derived from NPL12/Dular was also used to study the inheritance of pF18.

A reciprocal cross between the parents NPL12 and Dular was made to determine the pattern of inheritance. The IR32364TGMS and its original fertile breeding line IR32364 were also crossed with IR68 reciprocally. The F_1s of these reciprocal crosses were used to examine the inheritance of pF18.

DNA isolation and Southern analysis

Total DNA from the parents, F_2 individuals of the TGMS cross-combination, constituent lines and parents of the mapping population and F_1s of reciprocal crosses were extracted from fresh leaf tissues following the method of Dellaporta *et al*. (1983). Digested DNA was electrophoresed in a 0.9 per cent agarose gel, transferred to Hybond N+ membrane and analysed by Southern analysis as described by Sambrook *et al*. (1989).

PCR amplification of cob2 *and* nad1 *gene segments*

After confirming the sequence homology between the sequences of pF18, *cob2* and *nad1* of rice and wheat, respectively, we wanted to know whether the whole *cob2* and *nad1* were integrated in the TGMS line. PCR primers were designed such that one primer annealed within pF18, and the other primer was based on sequences in GenBank. For *cob2*, we synthesized the forward primer, cob2F, 5′-TCTCCTTTCCAAAGCTCCAC-3′, based on the sequence in GenBank Acc. no. X53711 and the reverse primer, cob2R, 5′-TAAATCCTAGTG-GAACCCGG-3′, based on the DNA sequence of pF18. The expected size of the PCR product was 1470 bp. For *nad1*, the forward primer, nad1F, 5′-TCTTCCCCTAACCCAACCCG-3′, was synthesized based on the pF18 sequence whereas nad1R, 5′-GCTGGGTAGGTTTGGCTATT-3′, was based on the wheat *nad1* gene sequence (GenBank Acc. no. X57967). The expected PCR product was 2183 bp. The PCR amplifications were carried out following the method of Hittalmani *et al*. (1995).

The PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C 30 s, 55°C 30 s, and 72°C 60 s with a final extension at 72°C for 5 min.

Results

Linkage confirmation between tms3 *and pF18 via Southern analysis*

In the population developed from the cross between IR32364TGMS and IR68, four DNA bands were detected in the sterile parent, IR32364TGMS, whereas only three bands were detected in the fertile parent, IR68, when the DNA was digested with *Eco*RV and probed with pF18 (Fig. 1a). The 3.3 kb band in the sterile parent cosegregated with *tms3* (Fig. 1b). All fertile homozygotes lacked the 3.3 kb band whereas all sterile plants had the 3.3 kb band. We therefore concluded that at least one band, detected by pF18, was linked to *tms3* and the band was in the nuclear genome as it segregated in the F_2 population.

Distorted segregation or maternal inheritance detected by pF18

In order to map the *tms3*-linked marker, pF18, a doubled haploid mapping population consisting of 135 lines derived from the cross IR64 × Azucena was used (Guiderdoni *et al*., 1992). An RFLP map comprising 135 markers has been constructed (Huang *et al*., 1994) and used to map RAPD markers linked to genes of agronomical importance (Zhang *et al*., 1996). Polymorphism was detected in the parents by Southern blot analysis. However, the progeny survey indicated a complete bias in the segregation pattern in favour of the *indica* (IR64) allele (data not shown). We were puzzled by the strong segregation distortion of the pF18 marker, and assumed that the segregation distortion might be caused by the selection effect of anther culture. To avoid segregation bias resulting from anther culture, an F_2 population derived from a cross between Tesanai 2 and CB (Lin *et al*., 1996) was used. Similarly, strongly skewed segregation in

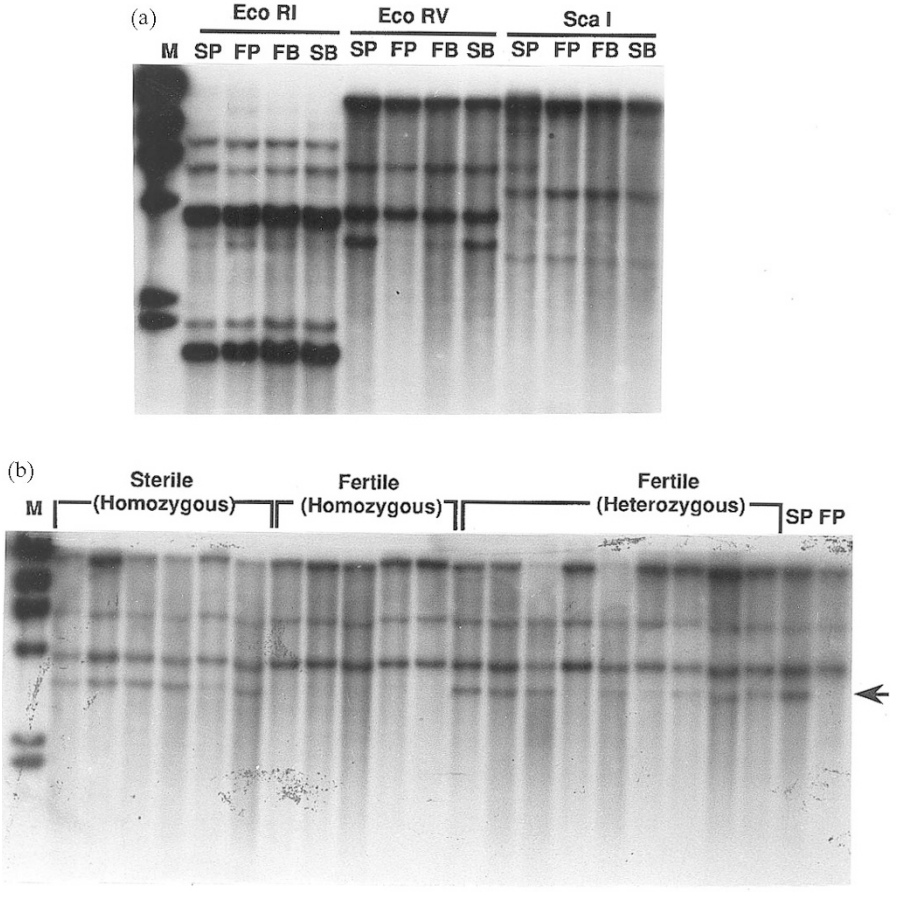

Fig. 1 (a) An autoradiogram showing bulked segregant analysis using pF18 as the probe. Three enzymes, *Eco*RI, *Eco*RV and *Sca*I, were used for digestion. Only in the case of *Eco*RV is a 3.3 kb band evident in both the sterile parent and the sterile bulk. (b) Southern blot analysis of *Eco*RV-digested F_2 progenies of the cross IR32364TGMS × IR68 using pF18 as the probe. The 3.3 kb band (indicated by an arrow) is present only in sterile and segregating fertile individuals indicating its dominant behaviour. M, molecular weight marker (Lambda/*Hin*dIII); SP, sterile parent IR32364TGMS; FP, fertile parent IR68; FB, fertile bulk; SB, sterile bulk.

favour of the Tesanai 2 allele was observed (Fig. 2). We attempted a third F_2 population derived from a cross between Norin PL12 and Dular but the same strong bias of segregation was also found (data not shown).

As segregation distortion is common in *indica* × *japonica* crosses (Ikehashi & Araki, 1988; Lin *et al*., 1992), we thought that pF18 might be tightly linked to a gene responsible for strong segregation distortion. Several such genes are already known in rice (Kinoshita, 1995). On the other hand, we also noticed that the bias in all three mapping populations was towards the female parents, implying maternal inheritance. Thus we hypothesized that pF18 might be originally derived from the cytoplasmic genome but transferred to the nuclear genome in IR32364TGMS. The marker sequence was therefore homologous to both nuclear (Fig. 1b) and organellar genomes. If this were true, Fig. 2 actually revealed cytoplasmic inheritance rather than strong segregation distortion.

Maternal inheritance confirmed in reciprocal crosses

The hypothesis of cytoplasmic (maternal) inheritance can be tested in reciprocal crosses. We analysed the reciprocal crosses involving the parents Norin PL12 and Dular (Fig. 3). All three maternal bands from Norin PL12 were present in F_1 individuals of Norin PL12 × Dular whereas only the Dular band was present in all the F_1 of Dular × Norin PL12. Maternal inheritance of pF18 was clearly evident.

Reciprocal crosses of IR32364TGMS × IR68 were also analysed. Fig. 4 shows the inheritance of the 3.3 kb *Eco*RV polymorphic fragment. The fragment was present in both crosses. Identical results were

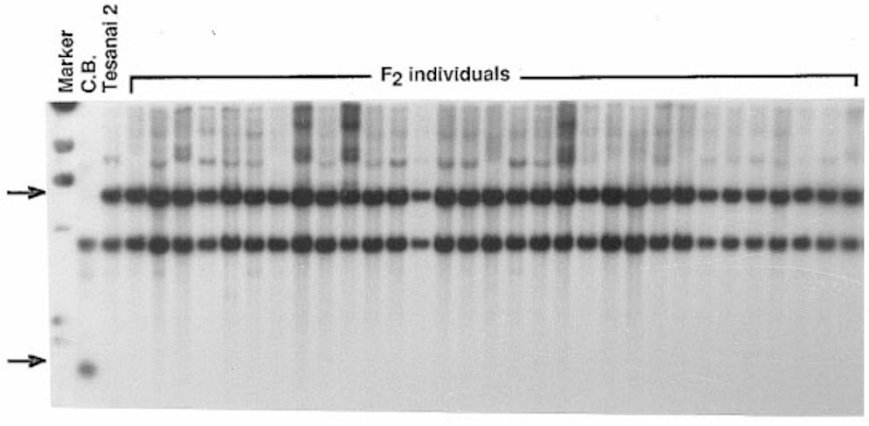

Fig. 2 Southern blot analysis of *Eco*RI-digested Tesanai 2 × CB F_2 individuals using pF18. Tesanai 2 and CB alleles are indicated by arrows. Only Tesanai 2 alleles are inherited in all individuals.

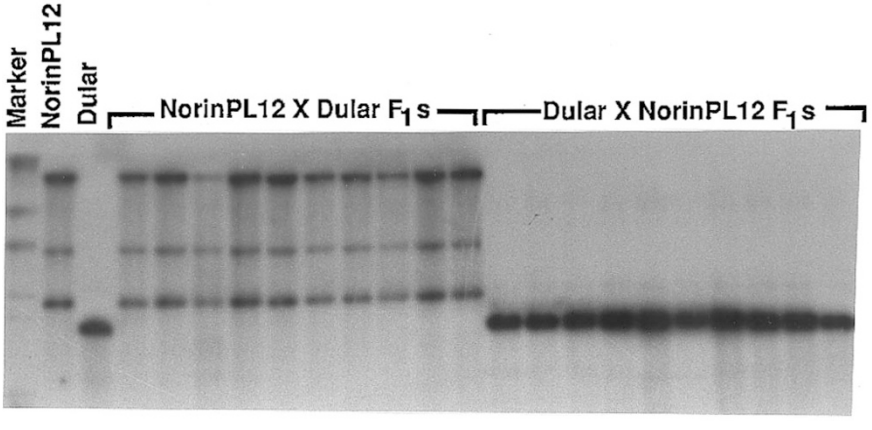

Fig. 3 Southern analysis of *Eco*RV-digested total genomic DNA derived from F_1 s of reciprocal crosses of Norin PL12 × TDular using pF18 as probe.

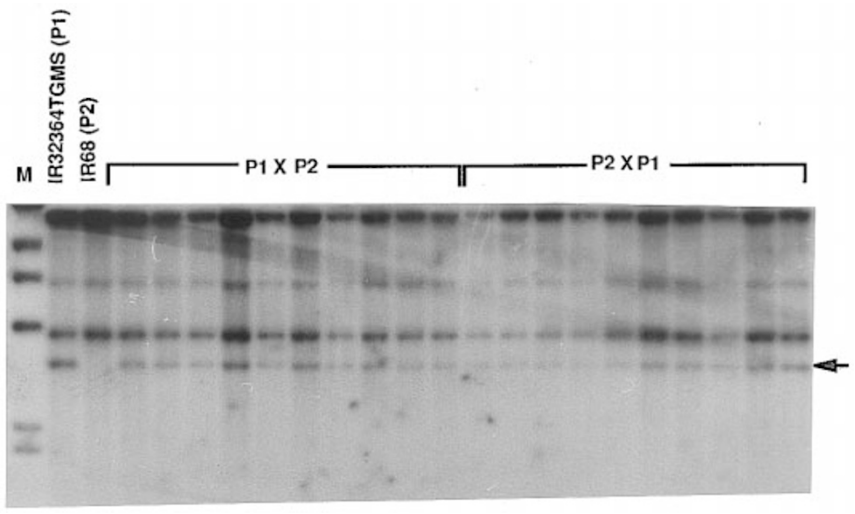

Fig. 4 Southern analysis of *Eco*RV-digested total genomic DNA in reciprocal crosses of IR32364TGMS × IR68 using pF18 as probe.

also obtained in reciprocal crosses of IR32364 × IR68 (data not shown). This clearly indicates nuclear inheritance of pF18 in the TGMS mapping population as well as in IR32364, the original fertile line used for mutagenesis. It can therefore be concluded that the pF18 marker loci were present in both nucleus and cytoplasm.

pF18 contains a mitochondrial DNA sequence

As pF18 was homologous to a DNA sequence in the cytoplasm, it was interesting to know if it was in the chloroplast or mitochondrial genomes. The pF18 was partially sequenced from both ends. A DNA sequence of 677 bp was obtained from the *Eco*RI end of the clone and named PF18F (Fig. 5a). Similarly, a DNA sequence of 626 bp was obtained from the *Xba*I end of the insert and named PF18R (Fig. 5b). A homology search of GenBank revealed that pF18 was highly homologous to the mitochondrial genes of many species. The rice pseudo-apocytochrome (*cob2*) gene (Acc. no. X53711) was homologous to PF18F, whereas PF18R was homologous to the *nad1*b, *1*c gene of wheat (Acc. no.

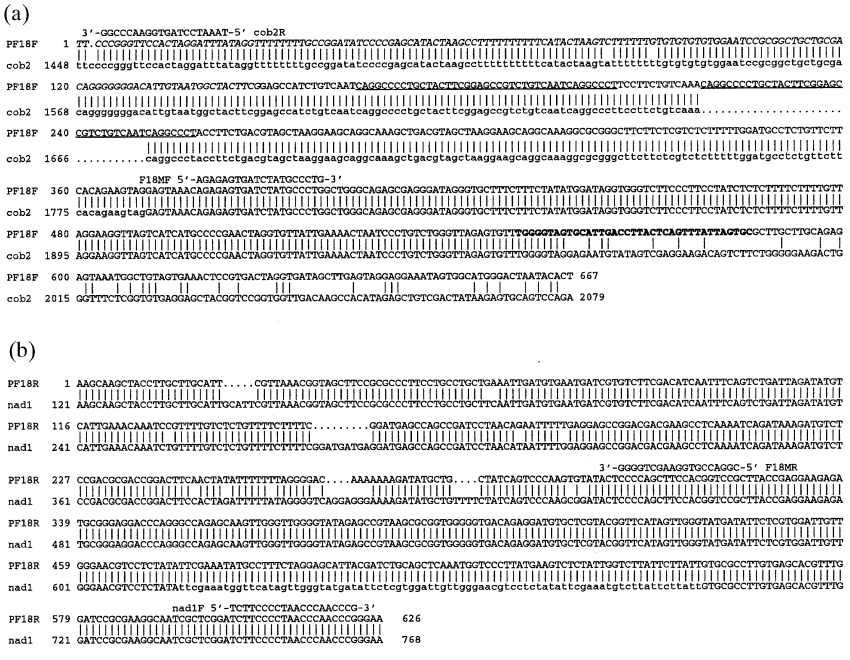

Fig. 5 (a) Sequence alignment of PF18F with rice pseudo-apocytochrome b (*cob2*) gene (Acc. no. X53711). Duplicated sequences are underlined. The 192 bp conserved sequence of *cob2* is italicized. Sequences in bold letters match B4 plasmid-like sequence (Fukuchi *et al*., 1991). (b) Sequence alignment of PF18R with the wheat *nad1* gene segment (Acc. no. X57967). The coding region is shown by lower case letters in the *cob2* and *nad1* sequences. Annealing position of primers for sequencing and PCR are indicated.

X57967), and *nad1*-like sequences of different species such as maize (Acc. no. M18339), petunia (Acc. no. X60401), and watermelon (Acc. no. X04130).

The sequence alignment of the *cob2* and *nad1* segments with pF18 is shown in Fig. 5. From Fig. 5(a) it is evident that PF18F was highly homologous to the rice *cob2* gene but the sequences diverged drastically after the 550 bp position. Within the homologous region, a 33 bp duplication was noticed in PF18F. A few features of PF18F are worthy of comment. First, the sequence of PF18F from positions 1–370 corresponded to a coding region and the stop codon in *cob2* indicating that PF18F contained both coding and noncoding regions. Secondly, PF18F contained the 192 bp conserved sequence of the *cob2* genes (Narayanan *et al*., 1995). Thirdly, a 37 bp sequence spanning homologous and nonhomologous regions of PF18F completely matched the B4 plasmid-like sequences which had been shown to transfer from mitochondrion to nucleus (Fukuchi *et al*., 1991).

The sequence of PF18R was highly homologous to the *nad1*b and *1*c gene segments of the wheat mitochondrial genome (Fig. 5b). The sequences from the 475–557 bp positions of PF18R corresponded to the first exon of *nad1*. The rest of the sequences in the PF18R segment were introns (Fig. 5b). Because *cob2* and *nad1* were commonly present in the mitochondrion, it was concluded that pF18 was transferred from mitochondrial to nuclear genomes.

Linkage of cob2 *with* tms3

If pF18 were transferred from mitochondrion to nucleus, and if the transferred fragment included entire *cob2* and *nad1* genes, then *cob2* and *nad1* should link to *tms3* as well. To test this hypothesis, PCR primers were synthesized based on sequences of GenBank for both genes and sequence of pF18 (see Materials and methods for detail). PCR products of the expected size (1470 bp) were obtained for *cob2*, whereas there was limited amplification of low molecular weight fragments for *nad1* (data not shown), therefore PCR analysis in segregating populations was conducted for *cob2* only.

The PCR analysis was first analysed with the doubled haploid lines of the IR64/Azucena population (Fig. 6a). A PCR band from IR64 is slightly larger than that from Azucena, showing codominant polymorphism. The progeny survey showed that all DH lines carried the band from the female parent, indicating that the *cob2* was inherited in maternal fashion in the IR64/Azucena population and the PCR products must be amplified from mitochondrial DNA.

In the IR32364TGMS × IR68 population, two bands were amplified from IR32364TGMS DNA, with the top band stronger than the lower one, whereas only one band was obtained with DNA from IR68 (Fig. 6b). Comparing the PCR bands from both parents, we found that one band was in common. As we know there is no gene insertion in IR68, the common bands in IR68 and IR32364TGMS must be derived from the mitochondrion. The other band in IR32364TGMS produces the dominant-type polymorphism as pF18. A survey of this band among sterile (with *tms3*) and fertile (without *tms3*) plants showed that all sterile plants

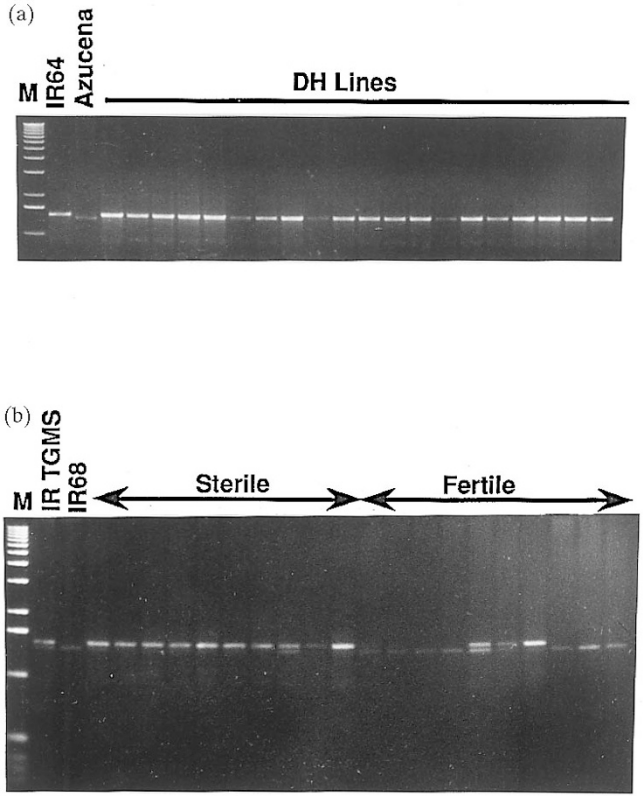

Fig. 6 PCR amplification of the *cob2* gene from total genomic DNA of rice. (a) PCR product from IR64 and Azucena showed two different alleles of *cob2* with a small difference in molecular weight. Maternal inheritance is clearly evident because of the amplification of only the IR64 (female parent) allele. (b) Two *cob2* alleles were present in IR32364TGMS whereas only one allele was present in IR68. PCR amplification of the *cob2* gene was conducted for the homozygous sterile and homozygous fertile F_2 individuals. M, molecular weight marker, kb ladder.

carried the dominant band, whereas only three of the 10 fertile plants carried the dominant marker. This demonstrated that *cob2*, like pF18, is also linked to *tms3* (Fig. 6b).

Discussion

Three lines of evidence have been provided that pF18 was derived from the mitochondrion: (i) pF18 was maternally inherited in three different populations (a doubled haploid and two F_2 populations) involving six different varieties; (ii) the maternal inheritance of pF18 was further confirmed by the observation of uniparental inheritance in reciprocal crosses; and (iii) the DNA sequence of pF18 was highly homologous to mitochondrial genes (*cob2* and *nad1*) of several plant species. It was therefore concluded that pF18 was transferred from the mitochondrion into the nuclear genome. A mapping study indicated that the insertion point was on the short arm of chromosome 6 (Subudhi *et al*., 1997).

There are several interesting features about this insertion. The first is that the transfer of pF18 was a DNA intermediate. Before our study, there had been ample evidence for transfer of genetic material from the mitochondria to nuclei documented in various flowering plants (see Fukuchi *et al*., 1991; Nugent & Palmer, 1991; Grohmann *et al*., 1992 for examples), showing flexible genetic information transfer among cellular compartments. Because the nuclear genomic sequences more closely resembled the edited sequences than the actual mitochondrial genes (Fukuchi *et al*., 1991; Nugent & Palmer, 1991; Covello & Gray, 1992; Grohmann *et al*., 1992), it was generally believed that an RNA intermediate was used during the transfer process. However, one report in *Arabidopsis* indicated involvement of a DNA intermediate (Sun & Callis, 1993). As pF18 contained exon, intron and intergenic sequences, we believe that pF18 was transferred via a DNA intermediate.

The second main feature of pF18 is its large size. Although the actual size of the insertion or the position of its borders are still unclear, the insertion includes both the 2.6 kb pF18 and 1.5 kb of *cob2*, so it is larger than 4.1 kb. To the best of our knowledge, this is the largest recorded DNA segment transferred from the mitochondrion into the nucleus. The insertion includes at least *cob2*, part of *nad1* and intergenic sequences. It remains to be clarified if the insertion is derived from a single continuous mitochondrial DNA segment or ligated DNA segments from several positions in the mitochondrial genome prior to DNA transfer.

The third interesting feature of the insertion is its genetic linkage to *tms3*. Furthermore, *cob2*, part of the insertion, has been shown to be associated with the wild abortive (WA) type cytoplasmic male sterility (cms) (Narayanan *et al*., 1995). Rearrangement of mitochondrial DNA has been demonstrated to be responsible for the T-type cms in maize. The mitochondrial gene, *T-urf13*, which is present in T cytoplasm but absent in normal cytoplasm, is responsible for the male sterility (Levings, 1993). Thus one might ask about the relationship between the pF18 insertion and cms or TGMS. Further study on the structure and function of the insertion might shed more light on this.

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