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ORIGINAL PAPER

Variations in the structure and transcription of the mitochondrial *atp* and *cox* genes in wild *Solanum* species that induce male sterility in eggplant (*S. melongena*)

M. Yoshimi · Y. Kitamura · S. Isshiki · T. Saito · K. Yasumoto · T. Terachi · H. Yamagishi

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Abstract In order to determine the molecular basis of cytoplasmic male sterility (CMS) in alloplasmic lines of eggplant, the genomic structures and transcription patterns of mitochondrial ATP synthase subunit (*atp*) and cytochrome oxidase subunit (*cox*) genes were studied for wild and cultivated eggplants. Alloplasmic eggplant lines with cytoplasms of wild *Solanum* species showing either anther indehiscent type of CMS or non-pollen production type of CMS were studied with the cultivated eggplant *Solanum melongena*, used as a control. Southern hybridization of the mitochondrial genes indicated the difference between the two types of CMS and showed complete identity within each type. The cytoplasmic patterns of all wild species differed from that of the cultivated eggplant. Thus, the cytoplasm of the six wild eggplants and the one cultivated eggplant was classified into three groups. Male sterile plants of both types of CMS showed novel transcription patterns of *atp1*, whereas a different transcription pattern of *cox2* was observed only in the anther indehiscent type. Based on these differences, we determined the DNA sequences of about a 4 kbp segment in the *atp1* region. Although the coding and 3′ flanking regions were almost identical among the cytoplasms, the 5′ flanking region was completely different and novel open reading frames (*orfs*) were found for each of the CMS types and the cultivated eggplant. The cytoplasm of *Solanum kurzii* inducing the anther indehiscent type CMS had *orf312*, and those of *Solanum aethiopicum* and *Solanum grandifolium* of non-pollen production type CMS had *orf218*. The correspondence between the transcription patterns of these *orfs* and phenotypic expression of male sterility strongly suggests that these *orfs* are causal genes for each type of CMS.

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M. Yoshimi · K. Yasumoto · T. Terachi · H. Yamagishi (✉)
Faculty of Life Sciences, Kyoto Sangyo University,
Kamigamo, Kita, Kyoto 603-8555, Japan
e-mail: hiyamagi@cc.kyoto-su.ac.jp

Y. Kitamura
Faculty of Agriculture, Shinshu University,
Minami-minowa 8304, Nagano 399-4598, Japan

S. Isshiki
Faculty of Agriculture, Saga University, Honjo 1,
Saga 840-8502, Japan

T. Saito
National Institute of Vegetable and Tea Science, Kusawa 360,
Ano, Tsu 514-2392, Japan

Introduction

Cytoplasmic male sterility (CMS) found in higher plants attracts scientific interest both from evolutionary and agronomical viewpoints. CMS has either arisen naturally in wild-plant populations as a result of mitochondrial recombination or been derived from intentional manipulation such as interspecific crosses (Laughnan and Newton 2012). The former type of CMS is considered to result from a series of recombination events in the mitochondrial DNA and substoichiometric shifting. The latter type is called alloplasmic CMS where usually cytoplasm of a cultivated plant is substituted with that of a wild relative species. While CMS is induced by a mitochondrial gene, the phenotypic expression is suppressed by a fertility restorer gene in nucleus. Thus, the CMS has been widely studied as the most fascinating example of the importance of interaction between mitochondria and the nucleus from two different

points of view, namely evolutionary genetics and plant breeding science (Budar and Berthomé 2007).

Because CMS is apt for reliable and efficient production of F_1 hybrid seeds, it has been used in many crops that exhibit hybrid vigor such as maize, onion, rice, sugar beets, and oilseed rape. Whereas, eggplant (*Solanum melongena*) shows hybrid vigor (Kakizaki 1931) and has the longest history of F_1 hybrid breeding in Japan (Saito et al. 2009). Eggplant is an important vegetable cultivated not only in tropical and subtropical but also in temperate Asian countries. Recently, the popularity of eggplant is increasing worldwide, and thus, higher productivity is required. However, CMS has not been exploited in eggplant so far. In order to produce hybrid seeds, it has been necessary to emasculate the seed parents and pollinate manually. These procedures are time consuming and labor intensive and are accompanied by the danger of accidental self-fertilization.

The substitution of cytoplasm of eggplant with that of the wild species of *Solanum* has previously been reported to result in alloplasmic male sterility. Until date, the cytoplasm of *Solanum gilo* (Fang et al. 1985), *Solanum violaceum* (Isshiki and Kawajiri 2002), *Solanum virginianum* (Khan and Isshiki 2008), *Solanum kurzii* (Khan and Isshiki 2009), *Solanum aethiopicum* (Khan and Isshiki 2010a), *Solanum anguivi* (Khan and Isshiki 2010b), and *Solanum grandifolium* (Saito et al. 2009) has been observed to function as a male sterile cytoplasm for eggplant. Furthermore, the phenotype of male sterility differs according to the type of cytoplasm. The male sterility with the cytoplasm of *S. violaceum*, *S. virginianum*, and *S. kurzii* is of the anther indehiscent type, whereas that with the cytoplasm of *S. aethiopicum*, *S. anguivi*, and *S. grandifolium* is of the non-pollen production type (Isshiki and Kawajiri 2002; Saito et al. 2009; Khan and Isshiki 2008, 2009, 2010a, b).

This differentiation in the phenotypic expression of alloplasmic male sterility led us to infer that there is variation in the cytoplasmic genomes of wild *Solanum* species. Although several wild species have been shown to induce CMS in eggplant, the genetic and molecular biological base behind this phenomenon have not been clarified. CMS is determined by the mitochondrial genome, and the causal gene of CMS often involves part of the ATP synthase subunit genes (*atp* genes) or the cytochrome oxidase subunit genes (*cox* genes), or it is located near one of these (Hanson and Bentolila 2004). Therefore, we examined structural variation around the mitochondrial *atp* and *cox* genes in wild *Solanum* species that have been ascertained to induce male sterility in eggplant. We identified variation in the mitochondrial genes that corresponded to interspecific differentiation and differences in the phenotype of male sterility in eggplant. This is the first report suggesting the causal genes of CMS.

Materials and methods

Plant materials

Male sterile progenies were obtained from interspecific hybridization of each wild species of *Solanum* (the seed parent) with the cultivated eggplant (*S. melongena*; the pollen parent) followed by successive backcrosses with the cultivated eggplant. The wild species used as cytoplasm donors included *S. violaceum*, *S. virginianum*, *S. kurzii*, *S. anguivi*, *S. aethiopicum*, and *S. grandifolium* (Table 1). When male sterile lines (Svio, Svir, Skur, Sang-b, and Saet-b; Table 1) of the former five species were used, the 'Uttara' cultivar was pollinated with each of the five wild species, and backcrosses were conducted with 'Uttara'. The backcross generations are shown in Table 1. On the other hand, S_{VF}-a and S_{VF}-b (Table 1) originated from 'Taibyo VF', a Japanese rootstock variety sold by Takii Seed (Kyoto, Japan). 'Taibyo VF' is an interspecific hybrid between *S. grandifolium* and *S. melongena*, and progenies were obtained by crossing with 'LS 1934' (*S. melongena*) (Saito et al. 2009). In this experiment, male fertile (S_{VF}-a) and sterile (S_{VF}-b) plants from the F_7 generation were studied.

Among the alloplasmic male sterile lines, Svio, Svir, and Skur demonstrated anther indehiscent type male sterility, whose phenotype was shown in Khan and Isshiki (2008). On the other hand, Sang-b, Saet-b, and S_{VF}-b showed non-pollen production type male sterility, where pollen is not observed at all as demonstrated in Saito et al. (2009). Because of restricted number of progeny plants derived from interspecific hybridization, we could not obtain male fertile lines for the anther indehiscent type, whereas male fertile lines were produced for the non-pollen production type male sterility by introduction of fertility

Table 1 Male sterile and fertile eggplants used in this study

Abbreviation	Origin of cytoplasm	Generation	Fertility
Svio	*S. violaceum*	BC_{10}	Sterile
Svir	*S. virginianum*	BC_4	Sterile
Skur	*S. kurzii*	BC_5	Sterile
Sang-a	*S. anguivi*	BC_5	Fertile
Sang-b	*S. anguivi*	BC_5	Sterile
Saet-a	*S. aethiopicum*	BC_4	Fertile
Saet-b	*S. aethiopicum*	BC_4	Sterile
S_{VF}-a	*S. grandifolium*	F_7	Fertile
S_{VF}-b	*S. grandifolium*	F_7	Sterile
Mel (*S. melongena* 'Uttara')	*S. melongena*		Fertile
Kur (*S. kurzii*)	*S. kurzii*		Fertile
Aet (*S. aethiopicum*)	*S. aethiopicum*		Fertile

restoration gene(s) [*Rf* gene(s)] from the wild parents. Thus, cytoplasm substitution lines showing pollen fertility (Sang-a, Saet-a, and S_{VF}-a) were included in this study. Moreover, as the control, the cytoplasm of the cultivated eggplant 'Uttara' (Mel) was studied. In addition, the expression of the mitochondrial genes of the fertile wild species *S. kurzii* (Kur) and *S. aethiopicum* (Aet) were compared with the CMS lines.

Southern hybridization of the mitochondrial genes

Total DNA was isolated from the young leaves of each of the male sterile and fertile eggplants and wild cytoplasm donors using a kit (DNeasy Plant Maxi kit; Qiagen, CA, USA). Five micrograms of total DNA was digested with each restriction enzyme of *Eco*RI, *Hin*dIII, and *Bam*HI, and the digest was electrophoresed in a 0.8 % agarose gel in 1× TAE buffer. The DNA fragments were then blotted onto a nylon membrane (Biodyne Plus; Pall, MI, USA). The blot was hybridized with four *atp* and three *cox* gene probes (*atp1*, *atp6*, *atp8*, *atp9*, *cox1*, *cox2*, *cox3*) labeled with digoxigenin (DIG) using a PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany). These probes were exploited by Leino et al. (2005), and were kindly provided by them. Hybridization (overnight in Roche's standard buffer at 65 °C) and post-hybridization washes (twice, for 15 min each, in 0.1× SSC, 0.1 % SDS, at 65 °C) were performed under stringent conditions.

RNA isolation and northern blot analysis

For northern blot analysis, total RNA was isolated from the flower buds (0.5–1 g) of male sterile plants with each type of CMS and from fertile plants as well as the parental wild species using a Sepasol (R)-RNA-I Super kit (Nacalai Tesque, Kyoto, Japan). About 15 μg of total RNA was heat-denatured and electrophoresed in a 1.2 % agarose gel containing 5.0 % (v/v) formaldehyde in 1× MOPS buffer. The RNA was then blotted onto a filter membrane (Biodyne Plus; Pall, Michigan, USA). The transcript pattern of each of the seven mitochondrial genes studied by Southern blot analysis was then examined by northern blot analysis. DIG labeling of the DNA probes, hybridization of the probes, post-hybridization washes, and chemiluminescent detection of the hybrid fragments were performed as described in the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

Nucleotide sequencing of the mitochondrial *atp1* region and exploitation of DNA markers to detect the CMS type

The DNA sequences of the coding and flanking regions of *atp1* were determined for Skur, Saet-b, S_{VF}-b, and Mel. Using the total DNA isolated from the leaves of each plant as the template, PCR was performed with the primer pairs listed in Table 2 and 25 μL of the 1× reaction buffer containing 0.5 U of KOD plus DNA polymerase (Toyobo, Osaka, Japan), 200 μmol/L each dNTP, 1.0 mmol/L $MgSO_4$, 0.3 μmol/L of the two primers, and template DNA. Amplifications were performed in a Veriti thermal cycler (Applied Biosystems, California, USA), which was programmed as follows: initial denaturation (94 °C, 2 min) followed by 30 cycles of denaturation (94 °C, 15 s), annealing (55 °C, 30 s), and extension (68 °C, 5 min).

For the coding region of *atp1*, PCR products were directly sequenced. The sequences of the 5′ and 3′ flanking regions were obtained by TAIL-PCR performed following the methods of Liu et al. (1995). The 5′ flanking regions of Skur, Saet-b, S_{VF}-b, and Mel were amplified by three nested PCRs using the arbitrary degenerate primer TAIL3 and 3 specific primers. The 3′ flanking regions of the four material plants were amplified by three nested PCRs using the arbitrary degenerate primer TAIL1 and 3 specific primers. Amplified fragments were cloned, and their sequences were obtained.

Based on the sequence data of the 5′ flanking region of *atp1*, probes for Southern hybridization and PCR primers were developed to distinguish the cytoplasm of wild and cultivated *Solanum* species. Because Skur, Saet-b, and Mel had different *orf* in the region from each other, three DNA proves were prepared within the *orfs*. Whereas the forward PCR primer was designed for the novel *orf* of each of the three cytoplasms, a common reverse primer with a DNA sequence associated with the *atp1* coding region was used. The DNA sequences of the primers are shown in Table 2. Southern hybridization was conducted as mentioned earlier. PCR was performed in a Veriti thermal cycler (Applied Biosystems) using KOD plus polymerase (Toyobo) with the following settings: initial denaturation (94 °C, 2 min) followed by 30 cycles of denaturation

Table 2 Primers and their sequences designed for sequencing of *atp1* region and for detecting the type of CMS in wild *Solanum* species

Primer	Site	Sequences (5′–3′)
PCR for sequencing of *atp1* region		
Satp1-Fw (forward)	*atp1*	GCGGCATTCTCTCTTATTTTC
Satp1-Rv (reverse)	*atp1*	CTTTGTGGTACCCCTAAGAG
PCR for detecting the type of CMS		
A (forward)	*orf312*	GAAGGAACCGCTATAATGACC
B (forward)	*orf218*	TGATCTTAGTGGTACTGTGC
C (forward)	*orf227*	CTTGGACCATTTCTACGTGT
D (reverse)	*atp1*	GTTATTCCTTTCACACCGCTGG

(94 °C, 15 s), annealing (55 °C, 30 s), and extension (68 °C, 2 min).

Results

RFLPs of the mitochondrial genes from the cytoplasm of *Solanum* species

Southern blot analyses were conducted for seven mitochondrial genes, five of which (except *atp9*, *cox1*) were polymorphic for the cytoplasm of the species studied in this experiment. The variation is demonstrated in Table 3, and exemplified in Fig. 1 for the probe of *atp1* gene. According to the combination of genes and restriction enzymes, the cytoplasms of the wild and cultivated species were classified into two or three types. The cultivated eggplant (Mel) possessed different RFLP types compared with the wild species. A correspondence between the type of male sterility and variation in the mitochondrial Southern hybridization patterns was observed. The three lines that showed anther indehiscent type male sterility (Svio, Svir, and Skur) had identical RFLP patterns for the five genes (Table 3; Fig. 1). Similarly, Sang-b, Saet-b, and S_{VF}-b, which had non-pollen production type CMS, demonstrated another RFLP pattern that was common to these cytoplasms alone (Table 3; Fig. 1). In total, the mitochondrial genomes analyzed here were classified into three types (Table 3) that showed correspondence with the CMS phenotypes. This is the first report to classify the mitochondrial genomes of eggplant and its wild relatives based on the induction of male sterility.

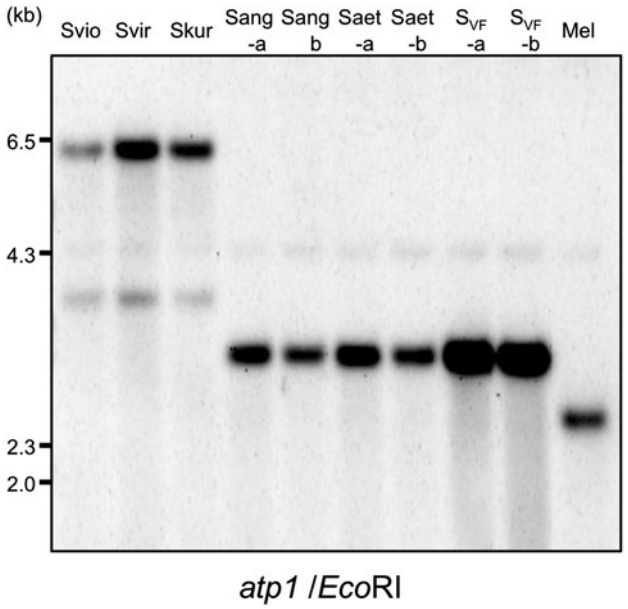

Fig. 1 Southern blot analysis of the mitochondrial *atp1* gene in alloplasmic eggplants with the cytoplasms of wild *Solanum* species. The DNA was digested with *Eco*RI. Svio, Svir, Skur, Sang-a, Sang-b, Saet-a, Saet-b, S_{VF}-a, S_{VF}-b and Mel; see Table 1

Transcription of mitochondrial genes in male sterile and fertile plants

Because the RFLP patterns of Svio, Svir, and Skur were identical but differed from those of Sang-b, Saet-b, and S_{VF}-b, which were also identical, we investigated the expression of mitochondrial genes using Skur as a representative of the anther indehiscent type of CMS and Saet-b

Table 3 RFLP patterns of the mitochondrial *atp* and *cox* genes in the cytoplasm of wild and cultivated eggplants

Genes	Restriction enzymes	RFLP type									
		Svio	Svir	Skur	Sang-a	Sang-b	Saet-a	Saet-b	S_{VF}-a	S_{VF}-b	Mel
atp1	*Eco*RI	I	I	I	II	II	II	II	II	II	III
	*Bam*HI	I	I	I	II	II	II	II	II	II	III
	*Hin*dIII	I	I	I	II	II	II	II	II	II	III
atp6	*Eco*RI	I	I	I	II	II	II	II	II	II	III
	*Bam*HI	I	I	I	II	II	II	II	II	II	III
	*Hin*dIII	I	I	I	II	II	II	II	II	II	III
atp8	*Eco*RI	I	I	I	I	I	I	I	I	I	II
	*Bam*HI	I	I	I	II	II	II	II	II	II	I
	*Hin*dIII	I	I	I	II	II	II	II	II	II	III
cox2	*Eco*RI	I	I	I	I	I	I	I	I	I	II
	*Bam*HI	I	I	I	I	I	I	I	I	I	II
	*Hin*dIII	I	I	I	II	II	II	II	II	II	III
cox3	*Eco*RI	I	I	I	I	I	I	I	I	I	II
	*Bam*HI	I	I	I	II	II	II	II	II	II	I
	*Hin*dIII	I	I	I	II	II	II	II	II	II	III

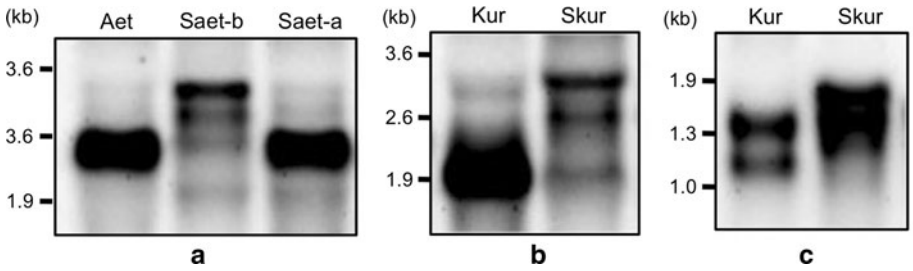

Fig. 2 Northern blot analyses of mitochondrial *atp1* (**a**, **b**) and *cox2* (**c**) in wild *Solanum* species and alloplasmic eggplants. Aet, Kur, Saet-a, Saet-b, and Skur; see Table 1

and S_{VF}-b as representative of the non-pollen production type of CMS. In addition to the above three lines, the following male fertile materials were compared with the CMS lines: Kur (*S. kurzii*,), Aet (*S. aethiopicum*), Saet-a, and S_{VF}-a.

Among the seven genes analyzed by northern blot analysis, difference in the size of the transcription products was found between the male sterile and fertile plants for two genes, *atp1* and *cox2*. The transcript of *atp1* in the male fertile wild species (Aet) was about 2.6 kb, but the alloplasmic male sterile line (Saet-b) possessed larger fragments (Fig. 2a). It is interesting to note that the male fertility restored line (Saet-a), which differed from Saet-b only with respect to the presence of fertility restoring gene(s), had an identical transcript to Aet. The difference in the transcript size of *atp1* between male sterile (S_{VF}-b) and fertility-restored plants (S_{VF}-a) was also observed for the cytoplasm derived from *S. grandifolium* (data not shown).

In case of Kur (male fertile) and Skur (male sterile), differences were observed in the *atp1* and *cox2* transcripts (Fig. 2b, c). The Skur transcription products from both genes were larger. Unfortunately, alloplasmic plant materials with fertility restoring gene(s) were not available for the CMS derived from Kur. Thus, we could not compare the sizes of the transcripts of *atp1* and *cox2* in a fertility-restored plant for the anther indehiscent type of CMS.

Nucleotide sequences of the *atp1* region and novel *orfs*

Based on the finding that the male sterile plants (Skur, Saet-b, and S_{VF}-b) of the two CMS phenotypes commonly demonstrated different transcription patterns of the *atp1* gene compared with fertile plants, we determined the DNA sequences of the coding and flanking regions of *atp1* in three male sterile plants as well as a cultivated one (Mel). DNA sequences of 3,912, 3,512, and 3,665 bp for Skur, Saet-b (and S_{VF}-b), and Mel, respectively, containing *atp1* were deposited in the DDBJ Nucleotide Sequence Database under accession numbers AB762696, AB762697, and AB762698. Among the materials studied, the sequences of Saet-b and S_{VF}-b, which had non-pollen production type CMS, were identical. All three sequences [Skur, Saet-b (and S_{VF}-b), and Mel] of the *atp1* coding site were 1,536 bp in size. While the sequences were highly similar to that of *Nicotiana tabacum* (1,530 bp), they were longer because the *atp1* coding sequences of the four *Solanum* cytoplasms possessed a "C" inserted at a location corresponding to the stop codon of *N. tabacum* (TAA → TCAA). This insertion induced a frameshift and read-through, resulting in the addition of DNA sequences coding two amino acids (TCA and ATT in place of TAA in *N. tabacum*) followed by the stop codon (TAA). Besides the frameshift elongating the coding region, the identical *atp1* genes of Skur, Saet-b, and S_{VF}-b contained 14 nucleotide substitutions relative to *N. tabacum*. The *atp1* of Mel had one nucleotide substitution compared with the other three *Solanum* cytoplasm; thus, it possessed 15 substitutions compared with *N. tabacum*. The nucleotide substitution in Mel from that of the other types of cytoplasm induced an amino acid replacement. The *atp1* of Mel and that of the cytoplasm of Skur, Saet-b, and S_{VF}-b, all of which had 511 amino acids, showed four and three amino acid replacements, respectively, compared with *N. tabacum* (509 amino acids). In addition, as described earlier, two amino acid insertions were observed at the 3′ end. The sequences of the three cytoplasm types were highly similar (86–88 %) to the nucleotide sequence of *N. tabacum* in the 3′ flanking region of *atp1*; they all possessed a sequence homologous (98–99 %) to that of exon 1 of *nad4* in *N. tabacum* downstream of *atp1*.

However, the 5′ flanking region of Skur, Saet-b (and S_{VF}-b), and Mel was completely different from that of *N. tabacum*. Furthermore, the three were different from each other, as shown schematically in Fig. 3. It was remarkable that all three sequences contained novel *orfs*: *orf312* in Skur, *orf218* in Saet-b, and S_{VF}-b, and *orf227* in Mel. More interestingly, although ORF227 was predicted to be a soluble protein, ORF218 and ORF312 were deduced to be transmembrane proteins from the DNA sequences. ORF218 possessed two hydrophobic domains, and ORF312 had four membrane-spanning domains in the N-terminal region. These facts indicate that ORF218 and ORF312 are pore-forming proteins, similarly with the products of various CMS genes studied so far (Budar and Berthomé 2007).

Next, we studied the transcription of *orf312* and *orf218* in male sterile and fertile plants with the cytoplasm from

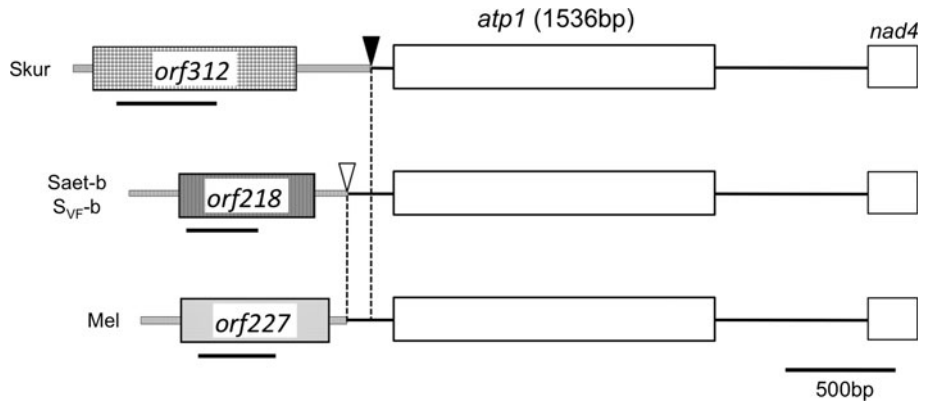

Fig. 3 Schematic structures of the flanking region of mitochondrial *atp1* in alloplasmic and cultivated eggplants. The region containing *atp1* and the downstream involving *nad4* is similar among the three cytoplasms. While the three cytoplasms have completely different sequence upstream the *atp1*, and they have novel *orfs*. The *black arrowhead* indicates the point where Skur is diverged from other two cytoplasms. The *white arrowhead* shows the differentiation point between Saet-b (S_{VF}-b) and Mel. The *horizontal bars* in the three *orfs* indicate the DNA probes for northern and Southern blot analyses of the *orfs*. Skur, Saet-b, S_{VF}-b and Mel; see Table 1

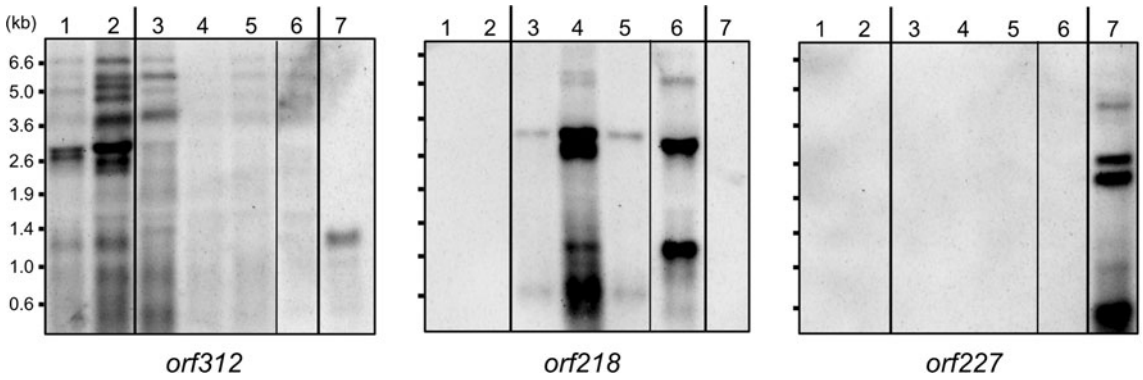

Fig. 4 Northern hybridizations of the novel *orfs* present in 5′ flanking region of mitochondrial *atp1*. DNA proves in the *orfs* shown in Fig. 3 were used. *1* Kur, *2* Skur, *3* Aet, *4* Saet-b, *5* Saet-a, *6* S_{VF}-b, *7* Mel (see Table 1)

the wild species by northern hybridization using a DNA probe for each of the *orfs* shown in Fig. 3. The mRNAs of *orf312* found in male sterile Skur were not detected in the fertile wild parent (Kur) (Fig. 4). Similarly, the transcripts of *orf218* were observed in the male sterile Saet-b plant, but the fertile wild parent (Aet) and the fertility-restored plant (Saet-a) lacked *orf218* transcripts (Fig. 4). Similarly, strong transcriptional signals were observed for *orf218* in the male sterile S_{VF}-b plant. Transcription of *orf227* was unique to the cultivated eggplant (Mel) (Fig. 4).

Southern blot analyses of *orfs* and PCR markers for detecting CMS type in wild *Solanum* species

The gel blot analysis with the DNA probe of *orf218* indicated that the three mitochondria inducing non-pollen production type CMS commonly had *orf218* regardless of the phonotype of male sterility (Fig. 5). On the other hand, the mitochondrial genome containing *orf312* was uniquely observed in the anther indehiscent type CMS (Skur, Svio, and Svir) (Fig. 5). The differentiation of mitochondrial genome into three types in the 5′ region of *atp1* was clarified similarly with other mitochondrial genes mentioned earlier (Table 1). From the results it was estimated that *orf218* and *orf312* were given rise to in a common ancestor species, respectively, by mitochondrial recombinations and were transmitted to the cytoplasms of wild species examined here. We are now determining the whole sequences of the three types of mitochondrial genome to know the origins of *orfs* in more detail.

In addition to the Southern blot analyses, a forward primer with the unique DNA sequences identified in each of the *orfs* and a common reverse primer with the DNA sequence from the *atp1* coding region was designed as shown in Table 2. PCR with the primer pair A–D produced a DNA fragment of the expected size in Skur, Svio, and Svir (Fig. 6a), the fact showing the three lines had *orf312* in the 5′ flanking region of *atp1*. PCR with the primer pair

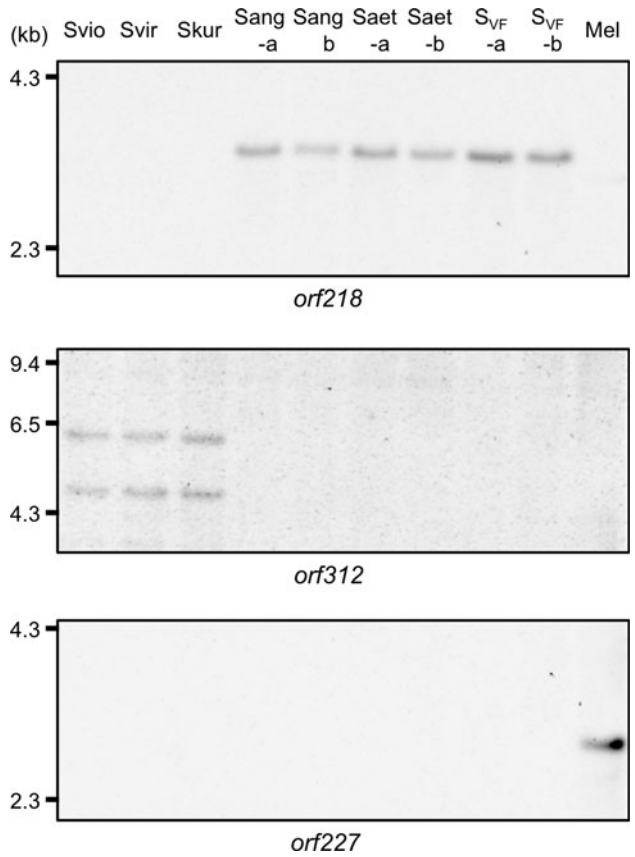

Fig. 5 Southern blot analysis of the novel *orfs* in 5′ flanking region of *atp1* in alloplasmic eggplants with the cytoplasms of wild *Solanum* species. The DNA was digested with *Eco*RI, and the proves in the *orfs* shown in Fig. 3 were used. Svio, Svir, Skur, Sang-a, Sang-b, Saet-a, Saet-b, S_{VF}-a, S_{VF}-b and Mel; see Table 1

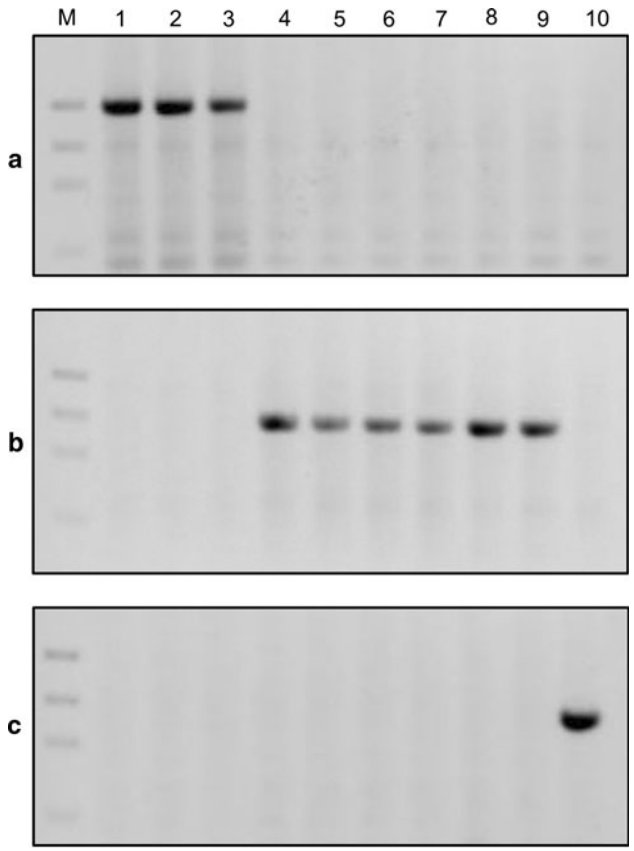

Fig. 6 PCR with the primer pairs designed for each of the novel *orfs* (forward) and the coding region of *atp1* (reverse). **a**, **b**, and **c** indicate PCR with the forward primer for A (*orf312*), B (*orf218*) and C (*orf227*) (Table 2), respectively. $M = \varphi$ X 174/*Hae*III, *1* Svio, *2* Svir, *3* Skur, *4* Saet-b, *5* Saet-a, *6* Sang-b, *7* Sang-a, *8* S_{VF}-b, *9* S_{VF}-a, *10* Mel (see Table 1)

B–D resulted in amplified fragments of the expected size in Saet-b, S_{VF}-b, and Sang-b (Fig. 6b). The result indicates that all three lines with non-pollen production type CMS possess *orf218*. In addition, the alloplasmic lines of Saet-a, Sang-a, and S_{VF}-a showed amplification of an identical fragment although they produce fertile pollens (Fig. 6b). In comparison with wild species, the cultivated eggplant (Mel) produced a DNA fragment only by PCR with the primer pair C–D (Fig. 6c). Figure 6 shows that the three lines with non-pollen production type CMS did not produce any PCR product with the primer pair A–D, whereas in the three lines with anther indehiscent type CMS, DNA amplification was not observed by PCR with the primer pair B–D. These PCR results indicate that the cytoplasm of the two types of CMS can be distinguished from each other and from the cultivated eggplant.

Discussion

The alloplasmic CMS of eggplants studied in this article was classified into two types, the anther indehiscent type of functional male sterility and non-pollen production male sterility. The phenotypic expression of both types of CMS has been examined under various environmental conditions over several years. All the plants used in this experiment have shown constant male sterility (Isshiki and Kawajiri 2002; Khan and Isshiki 2008, 2009, 2010a, b; Saito et al. 2009). Hence, the alloplasmic CMS of eggplants exploited until date is practically useful in F_1 seed production, and there is no danger of contamination by self-fertilized seeds.

Differentiation of the cytoplasm according to the type of CMS corresponded exactly to the differences in the structure of the mitochondrial genome revealed by Southern blot analysis. Most CMS is induced by the chimeric constitution of the mitochondrial genome as a result of recombinations of mitochondrial DNA, where a new open reading frame (*orf*) that involves or is adjacent to an existing functional gene such as one of the *atp* or *cox* genes is produced (Hanson and Bentolila 2004). *Orfs* are latent in an autoplasmic context; but, by substituting the nuclear genome, their phenotypic affection can be revealed by

pollen fertility. We examined the flanking regions of seven mitochondrial *atp* and *cox* genes, most of which have been reported to be involved in CMS. We identified differences in the Southern blot patterns of five genes from cytoplasm donors and the cultivated eggplant (*atp1*, *atp6*, *atp8*, *cox2*, *cox3*). Some of the differences in the mitochondrial DNA sequences flanking these five genes were considered candidate sites causative of CMS.

In order to study further the relationship between the variation in the mitochondrial genome and CMS, transcripts of the mitochondrial genes were observed by northern blot analysis. In the cytoplasm of *S. aethiopicum*, the male sterile plant (Saet-b) had a different size of transcript for the *atp1* gene compared with the fertile wild plant. However, the fertility-restored plant (Saet-a) showed the same transcript pattern as *S. aethiopicum*. Furthermore, similar differences in transcript size between the male sterile plant (S_{VF}-b) and fertility-restored one (S_{VF}-a) were found for S_{VF}. Such correspondences between changes in *atp1* transcript size and pollen fertility are important. We discovered a new and identical *orf* (*orf 218*) in the 5′ region of *atp1* in Saet-b and S_{VF}-b, and this may function as a gene causing CMS. In other words, the size difference indicates the presence or absence of normal processing of the co-transcript of *orf* and *atp1*, and the co-transcript leads us to two hypotheses. One is that *orf* is expressed and actively disrupts a mitochondrial function. Another is that appropriate activity of ATP synthase is suppressed by such co-transcription and results in the failure of normal production of fertile pollen. Northern blot analysis with a probe for *orf218* indicated that *orf218* is transcribed specifically in male sterile Saet-b and S_{VF}-b plants. Further, it is deduced that ORF218 is a pore-forming protein commonly with other known CMS gene products. These observations suggest that *orf218* is expressed and functions as a male sterile gene. To gain more knowledge about the mechanism, we are now investigating the accumulation of ORF218 and ATP1 proteins.

The alloplasmic CMS plants of anther indehiscent type had larger *atp1* and *cox2* mRNAs than the fertile wild *S. kurzii*. Because *atp1* was co-transcribed with a new *orf* in non-pollen type CMS as described above, we determined the DNA sequence of the *atp1* region for Skur and discovered a novel *orf* (*orf312*) that was also transcribed in the male sterile Skur plant. In Solanaceae plants, *orfs* found in the two gene sites (*atp1* and *cox2*) have been previously studied in relation to CMS. For example, *orf274* was found in the 5′ region of *atp1* in alloplasmic CMS of *N. tabacum* (Bergman et al. 2002), while *orf456*, which is present in the 3′ region of *cox2* in *Capsicum* has been postulated to be a causal gene of CMS (Kim et al. 2007). We, therefore, are currently attempting to determine whether a new *orf* is present in the flanking region of the *cox2* gene in Skur. In this respect, it is noteworthy that in several CMS plants, particularly in Solanaceae, an *orf* neighbors *atp1* in the mitochondrial genome and is co-transcribed with it. While *orf209* of the common bean and *orf214* of soybean are located in the 3′ region of *atp1* (Chase and Ortega 1992; Chanut et al. 1993), *orf274* of *N. repanda* is present upstream of *atp1* (Bergman et al. 2002). It should be emphasized that PCR with primers specific to *orf274* and *atp1* amplified DNA fragments in not only several *Nicotiana* species but also in *Petunia hybrida* and *S. tuberosum*, which belong to the same Solanaceae family as *S. aethiopicum* and *S. kurzii* (Bergmann et al. 2002). Therefore, it would be interesting to determine whether the co-transcription of *orf* and *atp1* is the common cause of male sterility in Solanaceae plants.

Besides the three plants for which novel *orfs* (*orf218* and *orf312*) were clarified (Saet-b, S_{VF}-b, and Skur), we also studied other wild species whose cytoplasm induced male sterility in the cultivated eggplant (Table 1). PCRs conducted with primers designed for *orf312* or *orf218* and the coding region of *atp1* demonstrated that all the plants with anther indehiscent type CMS commonly possessed *orf312,* whereas the plants with non-pollen production type CMS shared *orf218* (Fig. 6). It is notable that the cytoplasm of plants with the same CMS phenotype possessed a common *orf* in the 5′ flanking region of *atp1*. These findings suggest that *orfs* are causal genes of CMS in *Solanum* wild species. PCR with the primers designed for *orfs* was also useful for classification of the cytoplasm of the wild and cultivated *Solanum* species. (Although the RFLPs of the mitochondrial genes distinguished three types of cytoplasm in our study (Table 3; Figs. 1, 5), Southern blot analysis is technically demanding and time-consuming. While, PCR of the *atp1* 5′ flanking region efficiently discriminated three types of cytoplasm.)

For unambiguous identification of the CMS gene, it is necessary to study the expression of the candidate gene using a restored line to male fertility (Pelletier and Budar 2007). However, we did not produce any fertile alloplasmic progenies because of the small number of interspecific hybridization for the anther indehiscent type CMS. This would be a drawback for the identification of CMS genes although we identified *orf312* in the 5′ region of *atp1*. From a practical viewpoint of agriculture, however, *Rf* gene(s) are not always necessary for breeding F_1 hybrid eggplants. Rather, it has recently been determined that seedlessness is a desirable characteristic in eggplant because of the related sweetness of the fruit and good quality pickling (Khan and Isshiki 2009). Furthermore, parthenocarpic lines that do not require hormone treatment for fruit development have been established (Rotino et al. 1997; Kikuchi et al. 2008). The combination of CMS and parthenocarpic traits in future breeding will produce useful F_1 eggplants showing hybrid vigor and good quality.

In conclusion, in both CMS types, the normal processing of mRNA transcripts is disturbed in *atp1* by the substitution of the nuclear genome of the original wild species with that of the cultivated eggplant. Novel *orfs* were detected in the 5′ flanking region of *atp1*, and their unique transcripts corresponded with the male sterility phenotypes. Further observations of the accumulation of ORF proteins and their disappearance from plant tissues in accordance with phenotypic differences in male sterility and fertility would clarify their causal relationships with CMS.

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