Development of male sterile lines of eggplant (*Solanum melongena* L.) utilizing the cytoplasms of wild *Solanum* species

（ナス属野生種の細胞質を用いたナスの雄性不稔系統の育成）

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APPROVAL OF DISSERTATION

This dissertation entitled **"Development of male sterile lines of eggplant (*Solanum melongena* L.) utilizing the cytoplasms of wild *Solanum* species"** submitted to the United Graduate School of Agricultural Sciences, Kagoshima University, Japan, by **Md. Mizanur Rahim Khan** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Agricultural Science** is hereby approved on the recommendation of

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CHAPTER I

General Introduction

The *Solanaceae* is a very large plant family with 96 genra and 2,300 species, about one-half of which belong to the genus *Solanum* (D'Arcy, 1991). Several economically important species such as eggplant, potato, tomato, tobacco and pepper belong to the *Solanaceae* family. In the family *Solanaceae*, eggplant (*Solanum melongena* L.) is one of the non-tuberous cultivated herb. It belongs to the subfamily *Solanoideae*, the tribe *Solaneae*, the genus *Solanum*, the subgenus *Leptostemonum*, and the section *Melongena*. Eggplant, also known as aubergine, brinjal or Guinea squash, is of considerable economic importance in Asia, Africa, and subtropics (India, Central America), but is also grown in some warm temperate regions (Mediterranean area, South of USA) (Sihachakr et al., 1993). It is an autogamous diploid with 12 chromosomes (2n = 24). It is a good source of vitamins and minerals, especially iron, making its total nutritional value comparable with that of tomato (Kalloo, 1993). Besides being used as an important vegetable, eggplant has been exploited extensively in traditional medicines (Khan, 1979). For example, tissue extracts have been used for the treatment of asthma, bronchitis, cholera and dysuria; fruits and leaves are beneficial in lowering blood cholesterol. Recent studies have shown that eggplants also possess antimutagenic properties. The medicinal and economic value of eggplant can be found in the Sanskrit literature (Khan, 1979; Hinata, 1986; Kalloo, 1993). The world production of eggplant was estimated as 32 millions tons (t) from 2.04 milion hectares (FAO, 2007). Asia is the major eggplant producer, with China (18 million t) and India (8,5 million t) as leaders, followed far behind, by Egypt (1 million t), Turkey (800 000 t), Japan (380 000 t) and Italy (272 000 t).

The precise and definite places of the origin of eggplant have not been determined. Origin of eggplant was proposed to be India (Hara, 1944; De Candolle, 1984), China and India (Vavilov, 1926; Vavilov, 1951), South west Asia (Rolfs 1919), Asia (Khan, 1979) or African tropics (Lester and Hasan, 1991). The plant has been cultivated in Asia for over 1500 years. Arabs introduced this crop to the west during the fifteenth century (Hinata, 1986). Progressively, eggplant cultivation spread to the whole Mediterranean basin, Central Europe, Africa and then America and is cultivated worldwide today.

Plant improvement using conventional breeding methods has been directed towards fruit size, weight and shape, and resistance to diseases and pests (Kalloo 1993). This has led to the development of numerous varieties of eggplant with improved characteristics and yield. There are also many wild species of eggplant that are resistant to pests and pathogens and are the source of agronomically important genes that can be exploited for eggplant improvement (Collonier et al., 2001; Kashyap et al., 2003). Efforts to impart disease and pest resistance to cultivated varieties have been achieved with only limited success due to sexual incompatibilities with the source species or wild relatives (Daunay and Lester, 1988; Kashyap et al., 2003).

Biotechnological methods have been developed on eggplant for about a quarter of a century, and they are presently widely used in eggplant breeding processes. Eggplant tissues are easily grown in vitro, thus micropropagation, somatic embryogenesis, protoplast culture and protoplast hybridization, plant regeneration from cells or tissues, and anther culture are carried out successfully. Genetic engineering (transgenesis) is carried out since the 1980s and molecular mapping is developing since the end of the 1990s.

The exploitation of hybrid crop varieties in agriculture has enabled enormous increases in food productivity because of increased uniformity and hybrid vigour. Because of the hybrid vigour or heterosis, hybrids are characterized by increased resistance to diseases and enhanced performance in different environments compared with the heterozygous hybrid progeny (called F_1 hybrids) over the homozygous parents (Lefort-Buson et al., 1987). The increased vigour, uniformity and yield of F_1 hybrids have been exploited in most crops where the pollination system allows for economical and convenient cross hybridization (Basra, 2000). In hybrid seed production, one line is designated as the female parent and the other as the male parent. The production of hybrid seeds requires a pollination control system in order to prevent unwanted self-pollination of the female line. Especially for those crop species with hermaphrodite flowers, this can be a great challenge. To overcome this problem and ensure that crosses occur only between the selected female and male lines, several forms of pollination control have been used to produce hybrid seed in plants. These include (a) manual emasculation, (b) use of cytoplasmic male sterility (CMS) systems, (c) use of genic male sterility (GMS) systems, (d) use of self-incompatibility alleles, (e) use of male gametocides, and, more recently, (f) the use of genetically engineered pollination control systems.

Cytoplasmic male sterility (CMS), the maternally inherited trait of failure to produce or shed functional pollen has been extensively reviewed previously (Hanson, 1991; Schnable and Wise, 1998; Kempken and Pring, 1999). CMS systems represent a valuable tool in the production of hybrid seed in self-pollinating crop species, including maize, rice, cotton, and a number of vegetable crops. Cytoplasmically inherited male sterility (CMS) results from an interaction between the organellar and nuclear genomes that conditions the failure to produce functional pollen. In many

cases, the genetic basis for this trait has been traced to the mitochondrial genome. The association of CMS with abnormal mitochondrial gene expression has been established in many plant species including maize (Levings, 1990), petunia (Bino, 1985) and sorghum (Pring et al., 1995; Xu et al., 1995). It is thought that the disruption in pollen development is a consequence of mitochondrial dysfunction associated with the chimeric genes. Incorporation of the derived proteins into the mitochondrial membrane or into multiprotein enzyme complexes may lead to the impairment of mitochondrial function. Female fertility is generally not affected by CMS, so that male-sterile plants can and do set seed if viable pollen is provided. A unique feature of CMS is that expression of the trait is influenced by nuclear fertility restorer (*Rf*) genes (Schnable and Wise, 1998; Kempken and Pring, 1999). When nuclear fertility restorer (*Rf*) genes are available for CMS system in any crop, it is called as Cytoplasmic Genetic Male Sterility (CGMS). Nuclear fertility restorer genes can suppress or compensate the effect of the sterile cytoplasm and restore fertility to the next generation.

CMS has been observed in over 150 plant species (Laser and Lersten, 1972). Plant breeders have searched for such systems in many crop species; they have been successful in some, including cabbage, carrot, onion, radish, oilseed rape, rice, sorghum, sugar beet, sunflower and tobacco, for which large quantities of commercial F_1 hybrid seeds are currently produced using a CMS system (Havey, 2004).

Two sources of CMS are commercially used to produce hybrid onion. The most widely used source of CMS is S cytoplasm, as described by Jones and Clarke (1943). T cytoplasm is a second source of CMS (Berninger, 1965) used to produce hybrids in Europe and Japan (Harvey, 2000). CMS has also been described in chive (*A. schoenoprasum* L.) and Japanese bunching onion (*A. fistulosum* L.) and exploited for

hybrid production in both crops. CMS in chive shows unique sensitivity to tetracycline, which restores male fertility (Tatlioglu, 1986) and tetracycline susceptibility is conditioned by recessive alleles at a single locus (aa) (Tatlioglu and Wricke, 1988). CMS in Japanese bunching onion is conditioned by the male-sterile cytoplasm and recessive alleles at two nuclear restorer loci (Moue and Uehara, 1985). Alloplasmic sources of CMS conditioned by the cytoplasm of *A. galanthum* have been transferred to onion, shallot, and the Japanese bunching onion (*A. fistulosum* L.) (Havey, 1999; Yamashita and Tashiro, 1999; Yamashita et al., 1999). The main advantage of the galanthum CMS system is that nuclear male-fertility restoration loci appear to be rare or non-existent (Havey, 1999), indicating that many populations may be used to maintain this source of CMS.

Ogura (1968) identified CMS in radish, and this cytoplasm was transferred by backcrossing to *B. oleracea*. Unfortunately the Ogura CMS was associated with cold susceptibility, conditioned by the chloroplast genome. This defect was overcome by several different laboratories after protoplast fusion and organellar sorting to combine the CMS conditioned by the radish mitochondrial genome with cold tolerance conditioned by the Brassica chloroplast genome. A cold-tolerant form of Ogura CMS was patented by Syngenta and is used to produce hybrid Brassicas. Other companies and public labs (Walters and Earle, 1993) have developed similar cold-tolerant CMS lines independent of the Syngenta source.

For carrot, two sources of CMS are used to produce hybrid seed. The predominant CMS is the petaloid male-sterile cytoplasm, in which the anthers are replaced by a whorl of petals (Eisa and Wallace, 1969). The second source of CMS is brown anther, in which complete flowers produce shrivelled anthers with no pollen (Welch and Grimball, 1947). Male-fertility restoration for these sources of CMS is

complexly inherited with up to five loci affecting this trait (Peterson and Simon, 1986). Hybrid carrot represents approximately 50% of the world market, with petaloidy as the predominant source of CMS used for hybrid-seed production in the US (over 90%) and world (70%) markets.

CMS in chili peppers (*Capsicum annuum* L.) was found by Peterson (1958) in USDA accession PI 164835. The trait was found to be controlled by a major recessive *ms* gene interacting with a specific S plasmatype. A dominant *Ms* allele is necessary to restore pollen fertility. This CMS pepper was thought to be the only usable source for F_1 hybrid seed production using a cytoplasmic-genic male sterility system (Shifriss, 1997). Presently, a CMS system does not exist for the cultivated tomato, *Lycoperison esculentum*. In only one study, a CMS phenotype appeared as a result of the fusion of cytoplasmic inactivated tomato protoplasts with nuclear inactivated *Solanum* (*Solanum acuale* and *Solanum nigrum*) protoplasts (Melchers et al., 1992). Among regenerated fusion products female fertile plants with normal tomato characteristics were observed, but they completely lacked or had malformed anthers, had shrunken pollen, and pollen that did not germinate. The restriction analysis of mtDNA revealed that the mitochondrial genome of the CMS somatic hybrids did not combine all elements of the parental species and included new recombinant fragments.

CMS is used to produce hybrids of both table and sugar beets. The sole source of CMS used to produce hybrid beets was described by Owen in 1945. In beets, male sterility is conditioned by the interaction of the S cytoplasm with recessive alleles at two nuclear loci (xx zz). Owen (1945) recognized that the approach of Jones and Clarke (1943) could be used to produce beet hybrids. A second alloplasmic source of CMS from *Beta maritima* has been described (Boutin et al., 1987), but has not been used to date to produce commercial beet hybrids.

Engineered sources of nuclear male sterility have been developed in model systems (Mariani et al., 1990; Hernould et al., 1993; Perez-Prat and van Lookeren Campagne, 2002). A problem with these nuclear transformants is that they segregate for male fertility or sterility and must be over planted and rogued by hand or sprayed with herbicides to remove male-fertile plants. Nevertheless Brassica hybrid-seed is produced using the Bayer SeedLink^{tm} system, in which a transgene conditioning male sterility is linked to herbicide resistance. Hybrid Brassicas produced using the SeedLink^{tm} system have reached a significant market share by combining herbicide tolerance with consistent high performance.

Most of the intervarietal hybrids of eggplant are reported to have exhibited considerable vigor in economic characters, particularly the yield (Sambandam, 1962). For this reason, most commercial cultivars of eggplant are F_1 hybrids. In hybrid seed production of eggplant, the undesirable selfing and the necessity of labor-intensive manual emasculation (removal of anthers) are the two main quandaries. Emasculation of the seed parent, is time, labor and cost intensive. Incorporation of male sterile character in female parent lines could obviate emasculation in the hybridization process thus reducing the time, labor and cost for the hybrid seed production of eggplant.

Parthenocarpic fruits are seedless, a desirable trait in eggplant. The presence of seeds considerably deflates the value of fruits for both the fresh and the processed market. Seedless fruits of eggplant are generally more appetizing compared with seeded ones and liked by consumers. It is also yearn for improving the quality of pickled eggplants. There are two substitutes for the steady production of seedless fruits of eggplant. One is to spray flowers of male sterile eggplant with phytohormone and the other is to develop a good male sterility system of eggplant having

parthenocarpic character. For both ways, it is necessary to develop useful male sterile lines.

Male sterility in eggplant has been described in several reports. Functional male sterility by nuclear genes was reported by Jasmin (1954), Nuttall (1963), Phatak and Jaworski (1989) and Phatak et al. (1991). Jasmin (1954) first reported functional type of sterility in a population of Blackie eggplant developed from the cross Black Beauty by Black Nagasaki which was due to failure of the anthers to dehisce normally. Studies of the F_1 populations from male fertile and male sterile parents indicate that this character is recessive. Nuttall, (1963) obtained functional male sterility in the Ottawa eggplant mutant. From a test of three F_2 populations of different pedigrees showed that this trait inherited as a single recessive gene. Phatak and Jaworski (1989) described UGA 1-MS line derived from a spontaneous male sterile mutant of the cultivar Florida Highbush, the functional male sterility of which was due to a failure of anther dehiscence. Phatak et al. (1991) studied the inheritance of the functional male sterility in eggplant germplasm UGA 1-MS and its linkage with other characteristics. He reported the monogenic recessive control of this trait (gene fms), and its linkage with fruit purple colour. Chauhan (1984) reported a genic male sterility caused by malfunctioning of the tapetum and the sterility controlled by the interaction of two recessive nuclear genes designated as $ms_1ms_1ms_2$ ms_2. Although male sterility controlled by nuclear genes found in eggplant, they have not been utilized for the practical hybrid seed production of eggplant.

So far, two wild relatives of eggplant, *S. gilo* Raddi (Fang et al., 1985) and *S. violaceum* (Isshiki and Kawajiri, 2002) have been used to develop cytoplasmic male sterile lines of eggplant. Isshiki and Kawajiri (2002) obtained a cytoplasmic male sterility, expressed by no dehiscence of the anthers and low pollen fertility, in the allo-

plasmic backcross progenies obtained by repeated backcrossing of the interspecific hybrid between *S. violaceum* (female) and *S. melongena* (male) to *S. melongena.* This male sterility is suggested to be the result of a disharmony between the cytoplasm of *S. violaceum* and the nucleus of *S. melongena*. Fang et al. (1985) report two other cases of cytoplasmic male sterility in backcross progenies derived from the interspecific cross between *S. gilo* (i.e., *S. aethiopicum* Gilo Group) and *S. melongena*. One line had petaloid anthers, and the other had vestigial, pollenless anthers, both phenotypes being stable within the range of temperatures experimented. None of these lines has been utilized for the practical hybrid seed production of eggplant. Moreover, it is known fact that for a long-term commercially viable hybrid breeding program, both genetic as well as cytoplasmic diversity are essential. Utilization of a single male sterility source implies a potential risk because of the vulnerability of such a narrow genetic basis. Therefore, diverse sources of male sterility are strongly desired to widen this genetic base and to reduce the genetic vulnerability of the cultivated eggplant.

The conventional system of producing hybrid seeds through cytoplasmic male sterility is a time-consuming process that involves several years of repeated self-pollination and selection to develop the parental lines. In several species, this procedure is impeded by the necessity of creating CMS analogues through repeated backcrossing of the parental lines to the CMS donor to develop the CMS components (Welsh, 1981). Apart from backcrossing, the conversion of parental lines into their corresponding CMS components can be achieved by 1) protoplast fusion, a sophisticated method which cannot be generally applied (review Rose et al., 1990) because of the possibilities of chloroplast exchange and chloroplast and mitochondrial recombination (Rothenberg et al., 1985; Medgyesy et al., 1985) and 2) spontaneous androgenesis. Successful conversion of inbred lines into A lines has been

demonstrated in maize (Chase, 1963; Goodsell, 1961) and tobacco (Pelletier et al., 1987; Horlow et al., 1993) through spontaneous androgenesis. The main limitation however of the technique is the very low frequency of spontaneous haploids and the need for dominant morphological markers to identify haploids at the seedling stage (Genovesi, 1990). Anther culture is a system whereby the parental components of a hybrid produced through CMS are developed in a single generation.

Studies with anther culture have mostly been conducted for cultivated eggplant (*S. melongena*) with goal of obtaining double haploid parents for conventional breeding (Rotino, 1996). The double haploid plants have been successfully used in conventional breeding programs to obtain pure lines faster than selfed inbreds. Double haploid plants are homozygous at all loci, and this may help to study the genetic basis of quantitative traits by overcoming the problems associated with the envirobmental cariations. Raina and Iyer (1973) were first to report plant regeneration from anther culture in eggplant. They regenerated homozygous diploid (double haploids) plants through callus developed from anthers cultured at uninucleate pollen stage that were previously treated with colchicines. Haploid plantlets were also obtained from the Research group of Haploid breeding (1978) and Isouard et al. (1979) a year later. Dumas De Vaulx and Chambonnet (1982) did an extensive work to improve the development of androgenic haploids. They showed that high temperature (35 ± 2 ºC) incubation of anthers under dark conditions for the first 7-8 days improved the efficiency of haploid plant formation. A combination of both auxin and cytokinin was essential during early stages of anther culture. Similarly, Rotino et al. (1987) showed that haploid plant regeneration was affected by genotype, temperature, culture conditions, hormones and anther stage. A high temperature governs the shift of the microspores from gametophytic stage to sporophytic stage.

In this study, the present author paid attention to cytoplasms of the wild species as a CMS source. The purpose of this study is to investigate the possibility of developing male sterile lines of eggplant (*Solanum melongena* L.) utilizing the cytoplasms of wild *Solanum* species. The cytoplasms of *S. anguivi* and *S. kurzii* in section *Oliganthes* and *S. virginianum* in section *Melongena* were utilized to develop cytoplasm substitution lines of eggplant by continuous backcross method. This thesis comprises the following investigations: 1) Development of male sterile line of eggplant utilizing the cytoplasm of *S. anguivi* and their fertility studies, 2) Development of male sterile line of eggplant utilizing the cytoplasm of *S. kurzii* and their fertility studies, 3) Development of male sterile line of eggplant utilizing the cytoplasm of *S. virginianum* and their fertility studies, 4) Anther culture for producing pure lines of *S. virginianum* induced CMS line of eggplant.

This dissertation is a compilation of the results of the studies performed by present author at Department of Applied Biological Sciences, Faculty of Agriculture, Saga University, Japan, with the above-mentioned objectives from 2006 to 2008.

CHAPTER II

Development of a male sterile line of eggplant utilizing the cytoplasm of *S. anguivi* and their fertility studies

1. Introduction

S. anguivi Lam. like the cultivated eggplant, belongs to the subgenus *Leptostemonum*, but in different section *Oliganthes* and series *Aethiopica* of eggplant (Fig. 1). It is a wild progenitor of *S. aethiopicum* and originated in Africa (Lester et al., 1986). Serological and crossability studies (Pearce and Lester, 1979) have shown that *S. anguivi* is most closely related to egg-plant complex. Cultivated forms of *S. anguivi* are grown for their many small bitter fruits, which are used for medicine or for flavoring stews. The natural geographical distribution of *S. anguivi* ranges from East Africa both west and south (Lester et al., 1986). Weedy and semi-cultivated forms of *S. anguivi* have this same range, but are especially abundant in Ivory Coast and in Uganda. Material originating from Malagassy was called 'petit anghive' in French, which led Lamarck to name it *S. anguivi.* The wild, weedy, and semicultivated plants of *S. anguivi* are generally characterized by having prickles on the leaves and stems, a dense indumentum of stellate hairs on most parts, ten or more flowers in each inflorescence, and many small fruits, which are held up and are easily detached from the calyx. However, the cytoplasm of *S. anguivi* has not been utilized for the development of male sterile line of eggplant yet. In this study, the cytoplasm of *S. anguivi* was substituted for that of eggplant (*S. melongena* L.) by continuous backcross method to develop a new male sterile line of eggplant. Backcross progenies were examined for their pollen formation ability, pollen fertility, seed fertility, meiosis and organelle DNAs.

Fig. 1. *S. anguivi* (left) and *S. melongena* ‘Utara’(right).

2. Materials and methods

2.1. Plant material

To develop a cytoplasmic substitution line of eggplant, an interspecific F_1 hybrid (*S. anguivi* × *S. melongena* ‘Senryo Nigou’) was continuously backcrossed to *S. melongena* ‘Uttara’ using ‘Uttara’ as a recurrent pollen parent and *S. anguivi* as a cytoplasm donor, and five backcross generations, BC_1, BC_2, BC_3, BC_4 and BC_5 were produced.

In a process of the backcrossing, we were going to make F_1 hybrid (*S. anguivi* × *S. melongena* ‘Uttara’) first, however, did not succeed in making it. Therefore, we changed the male parent of the interspecific cross from ‘Uttara’ to ‘Senryo Nigou’ and succeeded in developing F_1 hybrid (*S. anguivi* × *S. melongena* ‘Senryo Nigou’). However, ‘Senryo Nigou’ was not used as recurrent pollen parent during the backcrossing since ‘Senryo Nigou’ is an F_1 cultivar.

In backcrossing, progenies were selected in the two directions of pollen non-formation and pollen formation types and both types of the backcross progenies at each generation were continuously backcrossed to ‘Uttara’, respectively (Fig. 2).

2.2. Pollen formation ability

Pollen formation ability was assessed by dissecting anthers at flowering day. Anthers from 15 flowers were examined for each plant.

2.3. Pollen fertility

Stainability of pollen with acetocarmine and *in vitro* germination rate of pollen were investigated for assessing the fertility of pollen. Pollen grains were extracted by

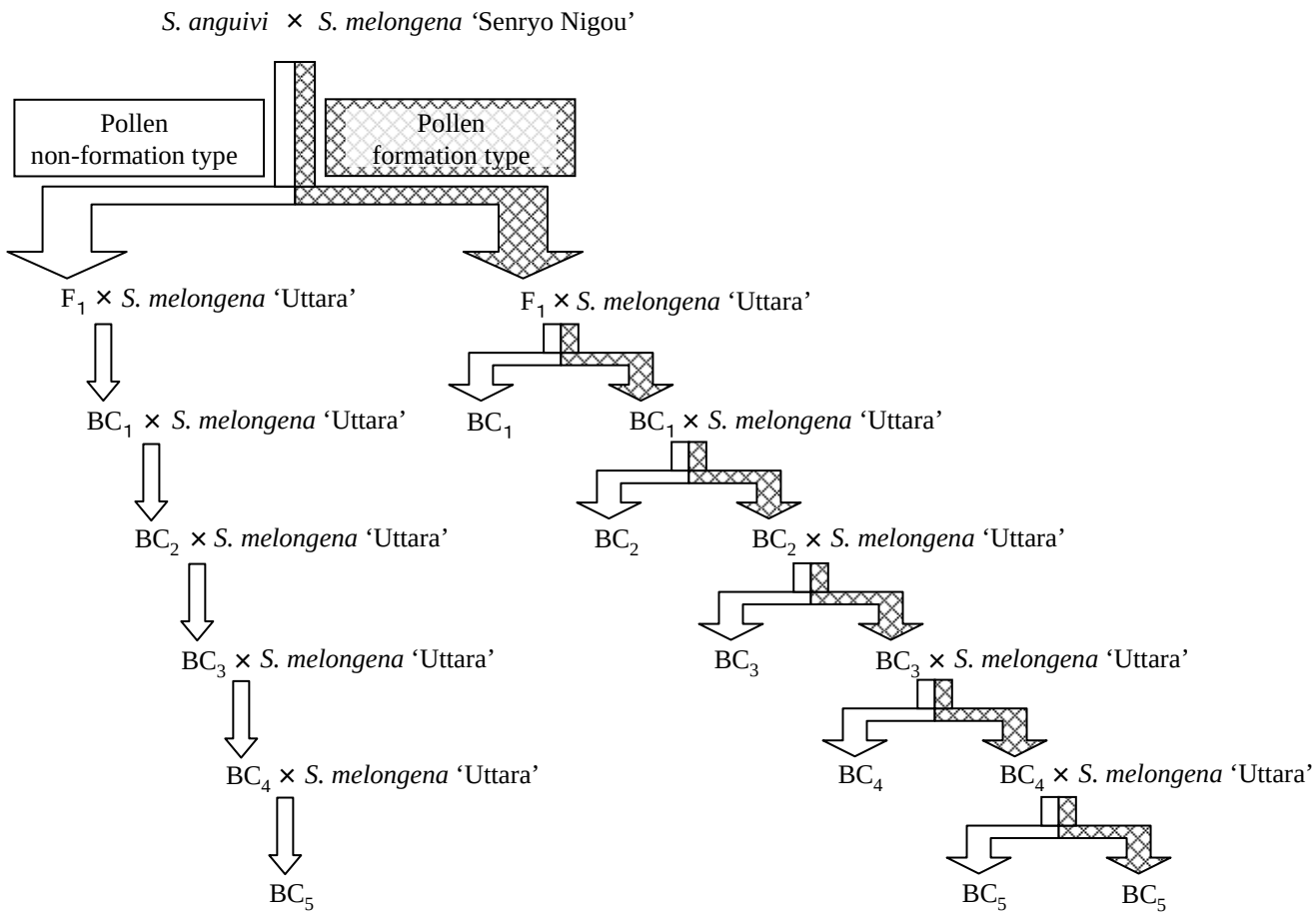

Fig. 2. Procedure for substituting the cytoplasm of *S. anguivi* for that of *S. melongena* 'Uttara' by continuous backcrossing.

Progenies were selected in the two directions of pollen non-formation and pollen formation from F_1 hybrid.

dissecting anthers from freshly opened flowers. Pollen stainability was determined by staining fresh pollen in a drop of acetocarmine solution. *In vitro* germination of pollen was investigated by using a germination medium consisted of 1% agar, 5% sucrose and 50 mg/l boric acid. Germination rate was determined after incubation at 25ºC for four hours. At least 500 pollen grains per flower were observed in 5 flowers per plant for assessing pollen fertility.

2.4. Seed fertility

Seed fertility of *S. anguivi, S. melongena* 'Uttara', the F_1 hybrid, and the BC_5 plants was estimated from the fruit set percentage, number of seeds per fruit and seed germination rate. The F_1 hybrid and the backcross plants were pollinated with *S. melongena* 'Uttara'. The *S. anguivi* and *S.melongena* 'Uttara' were selfed. For this investigation *S. anguivi, S. melongena* 'Uttara' and 2 F_1 plants and 4 BC_5 plants were examined. At least 10 flowers for each plant were hand pollinated. For seed germination, seeds were sown in soil in a glass house with controlled minimum and maximum temperatures of approximately 15ºC and 30ºC respectively. Seed germination was recorded 30 days after sowing.

2.5. Meiotic observation

Chromosome pairing at meiotic metaphase I in 30 pollen mother cells (PMCs) from fresh anthers of six plants of BC_5 progenies were observed by the smear method with acetocarmine.

2.6. Analyses of organelle DNAs

To identify the cytoplasm of backcross progenies, chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) were analyzed in *S. anguivi*, *S. melongena* 'Uttara' and 10 plants of BC_5 progenies. Total DNA was isolated from fresh leaves using the CTAB method described by Murray and Thompson (1980). Chloroplast DNA was analyzed by RFLP analysis of a PCR amplified region between *rbc*L and ORF106 following the method described by Isshiki et al. (1998). Sequences of the primers for the PCR amplification were 5'-ATGTCACCACAAACAGAAACTAAAGCAAGT-3' (*rbc*L) and 5'-ACTACAGATCTCATACTACCCC-3' (ORF106). The PCR product was digested with restriction enzyme *Rsa*I. Mitochondrial DNA was analyzed by RFLP analysis of a PCR amplified V7 region of mitochondrial small ribosomal subunit RNA gene. Sequences of the primers for the PCR amplification were 5'-TATGAACAACAAAACCTGTCTTTAACGGGATGG-3' ($mtV7_{P1}$) and 5'-GCGGACTTGACGTCATCCCCCACCTTCCTCCAG-3' ($mtV7_{P2}$) described by Buiteveld et al. (1998). The PCR product was digested with *Scr*FI. The digested PCR products both of cpDNA and of mtDNA were electroped by horesed on 1.5% agarose gel containing ethidium bromide and detected on a UV transilluminator.

3. Results

3.1. Pollen formation ability

For pollen formation ability, two types, i.e., the pollen formation and the pollen non-formation types were observed in the F_1 hybrid and the backcross progenies (Table 1). When the pollen non-formation type F_1 hybrid backcrossed to *S. melongena* 'Uttara', the following generations were fixed to the pollen non-formation type. The pollen non-formation type plants were completely devoid of pollen grains. However, when

Table 1. Segregation for pollen formation and pollen non-formation plants in the F_1 hybrid (*S. anguivi* × *S. melongena* 'Senryo Nigou') and the backcross progenies.

Plant materials	Number of plants			Chi-square (1:1)	$P_{df=1}$
	Total	Pollen formation	Pollen non-formation		
F_1	13	4	9	3.74	0.05-0.10
BC_1[a]	8	0	8	-	-
BC_2[b]	15	0	15	-	-
BC_3[b]	36	0	36	-	-
BC_4[b]	11	0	11	-	-
BC_5[b]	11	0	11	-	-
BC_1[c]	20	14	6	3.2	0.05-0.10
BC_2[d]	16	11	5	2.25	0.10-0.30
BC_3[d]	32	24	8	8	<0.01
BC_4[d]	22	16	6	4.55	<0.05
BC_5[d]	21	18	3	10.71	<0.01

[a] Obtained from seed parent of the pollen non-formation type F_1.
[b] Obtained from seed parent of the pollen non-formation type backcross progeny.
[c] Obtained from seed parent of the pollen formation type F_1.
[d] Obtained from seed parent of the pollen formation type backcross progeny.

the pollen formation type F_1 hybrid was backcrossed to *S. melongena* 'Uttara', segregation of the pollen formation and the pollen non-formation types occurred in all the backcross generations. Frequency of the pollen non-formation plants in all the backcross generations was found lower than that of the pollen formation plants. Segregation for the pollen formation and the pollen non-formation plants in BC_1 and BC_2 was not deviated from 1:1 ratio, however, segregation distortion from 1:1 ratio was observed in the succeeding backcross generations, BC_3, BC_4 and BC_5.

3.2. Pollen fertility

Pollen stainability and germination ability were very high in *S. anguivi* and *S. melongena* 'Uttara' (Table 2 and Fig. 3). Pollen stainability of the pollen formation F_1 and all the backcross generations was lower compared to both parental species. Interestingly, the stainability was nearly 50% in all the backcross generations. Pollen germination ability in the F_1 hybrid and all the backcross generations was very low.

3.3. Seed fertility

Fruit set percentage, number of seeds per fruit and seed germination rate were lower in the F_1 hybrid than *S. anguivi* and *S. melongena* 'Uttara' (Table 3). Fruit set percentage was high in all the examined BC_5 plants irrespective of pollen formation ability. Number of seeds per fruit in the BC_5 plants was moderately high although it was lower than that of *S. melongena* 'Uttara'. High seed germination rate was also found in the BC_5 plants.

Table 2. Pollen stainability and germination ability in the *S. anguivi*, *S. melongena* 'Uttara', pollen formation type F_1 hybrid (*S. anguivi* × *S. melongena* 'Senryo Nigou') and the backcross progenies.

Plant material	Pollen stainability (%)	Pollen germination ability (%)
S. anguivi	95.31	82.60
S. melongena 'Uttara'	95.44	85.46
F_1	17.05	5.24
BC_1	43.10±3.01 [a]	5.51±1.53
BC_2	45.87±3.18	3.47±0.69
BC_3	47.69±0.92	9.03±1.92
BC_4	55.52±1.67	10.42±1.64
BC_5	48.41±0.66	2.15±0.27

[a] Data are shown with mean ± SE.

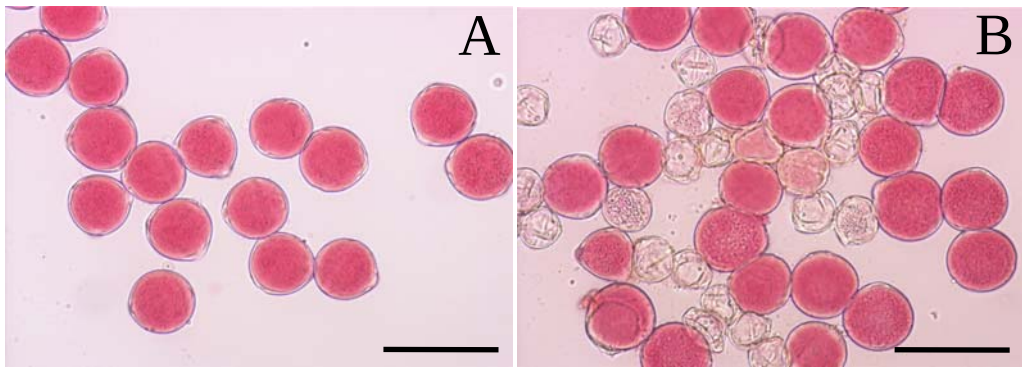

Fig. 3. Acetocarmine-stained pollen grains of (A) *S. melongena* ‘Uttara’ and (B) a plant of BC_5. Scale bar = 50 µm.

Table 3. Fruit set, number of seeds per fruit and seed germination rate in the *S. anguivi*, *S. melongena* 'Uttara', F_1 hybrid (*S. anguivi* × *S. melongena* 'Senryo Nigou') and the backcross progenies.

Plant material	Fruit set (%)	Number of seeds per fruit[a]	Number of seeds sown	Seed germination rate (%)
S. anguivi	100	56	100	63
S. melongena 'Uttara'	100	532	100	91
F_1				
1 (Pollen formation type)	20	16	45	49
2 (Pollen non-formation type)	7	13	26	38
BC_5				
1 (Pollen formation type)	100	59	100	-[b]
2 (Pollen formation type)	100	254	100	85
3 (Pollen non-formation type)	100	149	100	-
4 (Pollen non-formation type)	100	330	100	90

[a] Average of at least 7 fruits per plant except pollen non-formation type F_1 where average of 2 fruits is presented.
[b] Not investigated.

3.4. Meiotic observation

Meiosis in PMCs of the pollen formation BC_5 plants was usually normal. Two of the three BC_5 plants examined showed 12 bivalents in all the 30 PMCs examined at meiotic metaphase I (Fig. 4). The other one plant exhibited 12 bivalents in all the PMCs except the one which showed 11 bivalents and 2 univalents. After meiosis, the microspores were released from the tetrads and then some microspores degenerated resulting in the appearance of empty pollen grains in the anthers (data not shown). No meiosis was observed in the pollen non-formation BC_5 plants.

3.5. Analyses of organelle DNAs

Both of the pollen formation and pollen non-formation BC_5 plants examined had the restriction patterns identical to those of *S. anguivi* in the analyses of both cpDNA (Fig. 5) and mtDNA (Fig. 6).

4. Discussion

To substitute the cytoplasm of *S. anguivi* for that of *S. melongena* ‘Uttara’, fifth backcross generations were successfully produced by continuous backcrossing in this study. In the analyses of both cpDNA and mtDNA, all the BC_5 progenies examined had the restriction patterns identical to those of the cytoplasm donor *S. anguivi*. These results provide clear evidence for maternal inheritance of cpDNA and mtDNA in backcross progenies examined which confirm the successful substitution of the cytoplasm of *S. anguivi* for that of eggplant by continuous backcross method. Maternal inheritance of the present study is in accordance with most plants where organelle inheritance is strictly maternal (Reboud and Zeyl 1994).

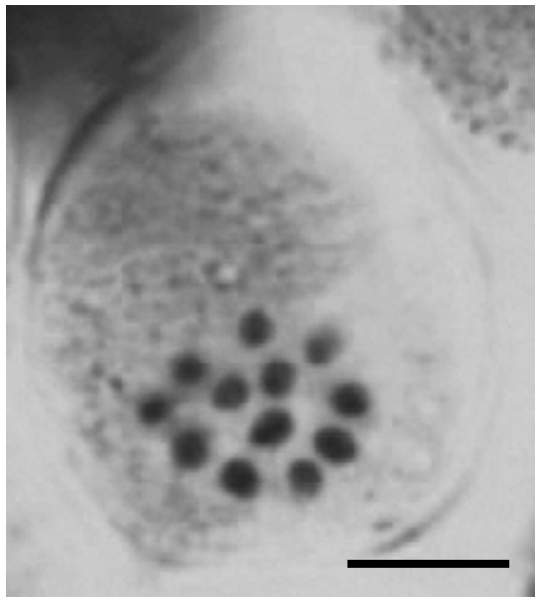

Fig. 4. Meiotic metaphase I in a pollen mother cell of a plant of BC_5 showing 12 bivalent formation. Scale bar = 10 µm.

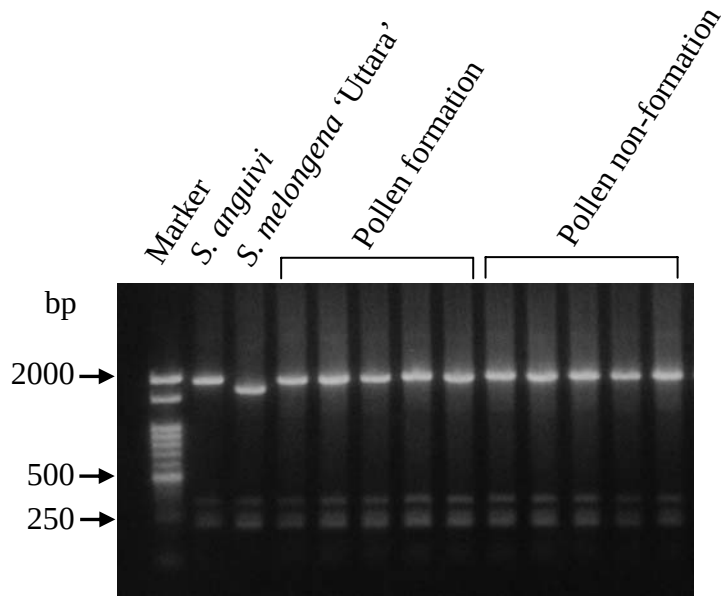

Fig. 5. Restriction patterns of the *Rsa*I digested *rbc*L-ORF106 region of cpDNA in *S. anguivi*, *S. melongena* 'Uttara', and the BC_5 plants of the pollen formation and pollen non-formation types.

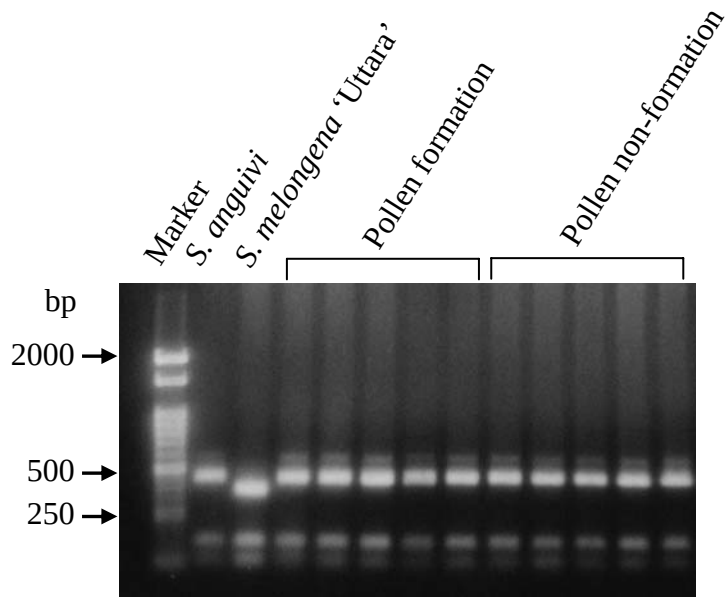

Fig. 6. Restriction patterns of the *Scr*FI digested V7 region of mitochondrial small ribosomal subunit RNA gene in *S. anguivi*, *S. melongena* 'Uttara', and the BC_5 plants of the pollen formation and pollen non-formation types.

Segregation patterns for the pollen formation and the pollen non-formation types in the backcross progenies indicate that a single dominant fertility restoring gene (*Rf*) controls the pollen formation of the *S. melongena* with the cytoplasm of *S. anguivi*, and that this *Rf* originated in the nuclear genome of *S. anguivi*. That is to say, it is presumed that the pollen formation type had the genotype *Rfrf* and the non-formation one had the *rfrf*, where the *rf* is a recessive fertility non-restroring gene. In the F_1 hybrids, segregation of the pollen formation ability was recognized and all the BC_1 plants obtained from the pollen non-formation F_1 hybrid were the pollen non-formation type, without segregation (Table 1). This indicates that the pollen non-formation F_1 hybrid had only the *rf* allele. The genotype of the parental *S. anguivi* used in the present study might be heterozygous (*Rfrf*) for the pollen formation ability.

The lower frequency of the pollen non-formation type was recognized in all the segregating backcross progenies (Table 1). Although the segregation ratio in the BC_1 and the BC_2 progneies was not deviated from 1:1 remarkably, the subsequent generations, BC_3, BC_4 and BC_5, showed clear segregation distortion from 1:1. Generally, segregation distortion may arise from reproductive barriers like hybrid sterility, differential viability of gametes, and hybrid weakness (Fukuta et al., 2006), chromosomal aberrations like directed chromosome loss, reciprocal translocation and inversions (Midro et al., 2006), lethal or sub-lethal genes that are associated with inbreeding depression and genetic load (Bradshaw and Stettler, 1994; Perfectti and pascual, 1996) etc. In the present study, clear segregation distortion was recognized in the BC_3, BC_4 and BC_5 generations in which most of the nuclear genes (more than 93 %) were occupied by those of *S. melongena*. Furthermore, almost complete 12 bivalents formed at meiotic metaphase I of PMCs in the pollen formation BC_5 progenies indicates quite normal meiosis of them (Fig. 2). Therefore, the segregation

distortion is suggested not to be attributed to any of reproductive barriers or chromosomal aberrations but the lethal gene which is associated with the *rf* gene. This lethal gene might be recessive and work in the lines of *S. melongena* with the cytoplasm of *S. anguivi*. For exact elucidation of mode of inheritance of the *Rf* gene and the putative lethal gene, genetic analyses using test cross progenies, such as, selfed ones of the pollen formation backcross progenies etc. should be imperative.

Both the pollen stainability and germination ability of the pollen formation type F_1 hybrid was quite lower than those in the parental *S. anguivi* and *S. melongena*. Similar results have been obtained for the F_1 hybrids between *S. melongena* and the related *Solanum* species (Rajasekaran 1970; 1971; Rangasamy and Kadambavanasundaram 1974; Nishio et al. 1984). Low pollen fertility in the present F_1 hybrid might be the result of cryptic structural differences in the parental chromosomes as in previously reported (Rajasekaran 1970; 1971).

Pollen stainability of the pollen formation plants in all the backcross progenies showed approximate 50% and there was no tendency for the stainability to increase with the subsequent backcross generations. As meiosis of PMCs in the pollen formation BC_5 progenies was quite normal, it is suggested that the approximate 50% pollen stainability was not due to meiotic difficulty but genetic segregation of the pollen genotypes. Schnable and Wise (1998) reported that in the case of a plant heterozygous for a gametophytic restorer, only those gametes that carry the restorer will be functional. Therefore, the mode of pollen stainabilty restoration in the present study might also appear to be gametophytic, namely, only the pollen grains with the *Rf* were stained whereas those with the *rf* were not. Further studies are essential to confirm this hypothesis.

High fruit set percentage, moderately high number of seeds per fruit and high seed germination rate found in the BC_5 progenies indicate that the cytoplasm of *S. anguivi* has no notable negative effect on seed fertility of the *S. melongena* with the cytoplasm of *S. anguivi*. The number of seeds per fruit in the BC_5 progenies was comparatively lower than *S. melongena*. More backcrossing with a selection of high seed fertility individuals would be effective for developing the useful CMS line of *S. melongena* with high seed fertility.

Cytoplasmic male sterility in *S. melongena* has been reported previously. The functional CMS line with the pollen non-release type with the cytoplasm of *S. violaceum* (Isshiki and Kawajiri 2002) and the petaloid and vestigial anther type CMS line with the cytoplasm of *S. gilo* Raddi (Fang et al. 1985) have been developed. In the present study, the pollen non-formation type CMS line could be developed. Therefore, the nuclear-cytoplasmic interactions on the male fertility of *S. melongena* seems to vary with differnece of the cytoplasms. Some male sterility systems have been reported to be influenced by the environment and insects (Phatak et al. 1991; McVetty 1997). As the CMS line developed in the present study showed the pollen non-formation type male sterility, there is no risk of pollen release from anthers resulting in stable expression.

The present study demonstrates that it is possible to develop a new male sterile line of eggplant by utilizing the cytoplasm of *S. anguivi*. It is necessary to widen the range of male sterility systems for their practical uses, such as hybrid seed production and seedless fruit production, in eggplant. The present male sterile line of eggplant with the cytoplasm of *S. anguivi* offers a valuable alternative.

CHAPTER III

Development of a male sterile line of eggplant utilizing the cytoplasm of *S. kurzii* and their fertility studies

1. Introduction

Taxonomic position of *S. kurzii* Brace & Prain (syn. *S. sanitwongsei* Craib.) (Fig. 7) is unclear. From the studies of isozymes (Isshiki et al., 1994), cpDNA (Isshiki et al., 1998) and mtDNA (Isshiki et al., 2003), *S. kurzii* showed identical results to that of *S. violaceum*, which belong to the same section *Oliganthes* series *Afroindica*. From ISSR analysis, Isshiki et al (2008) also confirmed close affinity of them. *S. kurzii* exhibited the same morphology of *S. violaceum*, except for the absence of prickle and assumed to be a prickle-free mutant of *S. violaceum* (Isshiki et al., 2003). The cytoplasm of *S. kurzii* has not been utilized for the development of male sterile line of eggplant yet. In this study, the cytoplasm of *S. kurzii* was substituted for that of eggplant (*S. melongena* L.) by continuous backcross method to develop a new male sterile line of eggplant. Backcross progenies were examined for their pollen release ability, pollen fertility, seed fertility, chromosome association at meiotic metaphase I (MI) and organelle DNAs.

2. Materials and methods

2.1. Plant material

An interspecific F_1 hybrid between *S. kurzii* and *S. melongena* 'Uttara' was made using *S. kurzii* as the female parent and eggplant 'Uttara' as the pollen donor.

Fig. 7. *S. kurzii* Brace & Prain.

Through repeated backcrossings to eggplant, using eggplant as the recurrent male parent, three backcross generations, BC_1, BC_2 and BC_3, were produced. Twelve BC_1 (single population), 22 BC_2 (two population), and 20 BC_3 (two populations) plants were used in the present study.

2.2. Pollen release ability

Pollen release ability was assessed by tapping the pore end of the anthers in open flowers onto a slide and observing the anthers by a stereomicroscope. Anthers from ten flowers of each plant were examined.

2.3. Pollen fertility

Pollen stainability and *in vitro* germination ability of pollen were investigated for assessing pollen fertility. Pollen grains from freshly opened flowers were extracted from the anthers by dissection, then smeared in 1% acetic carmine to assess their staining ability using the method described by Singh (2002). *In vitro* germination ability of pollen was investigated according to Singh (2002) with a slight modification following germination medium consisted of 1% agar, 5% sucrose and 50 mg/l boric acid. Pollen germination ability was determined after incubation at 25 ºC for four hours. More than 500 pollen grains were examined for each plant assessing pollen fertility.

2.4. Seed fertility

Fruit set percentages, and the number of seeds per fruit were counted to assess seed fertility. For this investigation, one F_1, four BC_1, four BC_2 and four BC_3 plants were pollinated using the male parental eggplant. The parental eggplant and *S. kurzii* were

also selfed. At least ten flowers on each plant were hand-pollinated. Germination rates of seeds obtained from four BC_3 plants at random selected and the parental eggplant were also investigated. One hundred seeds from each of the plants were sown in soil in a glasshouse, with minimum and maximum temperatures set at approx. 15˚C and 30˚C, respectively. Seed germination was observed 30 d after sowing.

2.5. Observation of chromosome association

Chromosome associations at meiotic metaphase I (MI) were observed in the 20 BC_3 plants, and in the parental eggplant, by smear preparations of pollen mother cells (PMCs) from fresh anthers in 1% acetic carmine (Nishiyama, 1961). At least 30 PMCs were observed for each plant.

2.6. Identification of cytoplasm

Cytoplasm from all BC_3 plants was identified by analyzing the chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) to confirm maternal inheritance from *S. kurzii*. Total DNA was extracted from fresh leaves of each plant using the CTAB method, as described by Murray and Thompson (1980). For cpDNA, PCR-RFLP analysis of the region bounded by the conserved sequences in *rbc*L and ORF106 was conducted following the method described by Isshiki *et al*. (1998). For mtDNA, PCR-RFLP analysis of the V7 region of the mitochondrial small ribosomal subunit RNA (srRNA) gene was performed using the method described by Yamashita *et al*. (2000).

3. Results

3.1. Pollen release ability

Solanum kurzii, *S. melongena*, and their F_1 progeny all released pollen. In the backcross progenies, plants which did not release pollen appeared, although all the backcross progenies produced pollen grains in their anthers, regardless of their pollen release ability (Table 4). All the anthers of the pollen "non-release-type" in the backcross progenies were of the indehiscent-type (Fig. 8). Dehiscence of some anthers and a slight release of pollen grains were observed in all pollen-release-type backcross progenies. BC_1 plants segregated into both anther-dehiscent- and indehiscent-types. All BC_2 plants whose maternal BC_1 parent was the anther-dehiscent-type, were the anther dehiscent type. All the BC_2 plants, whose maternal BC_1 parent was the anther-indehiscent-type, were also the anther-indehiscent-type, except for one plant. BC_3 plants, whose maternal BC_2 parent was the anther-dehiscent-type, were all the anther-indehiscent-type except two plants. BC_3 plants whose maternal BC_2 parent was the anther-indehiscent-type, were all of the anther-indehiscent-type, without segregation.

3.2. Pollen fertility

Pollen fertility in the F_1 and in all backcross generations, BC_1, BC_2, and BC_3, was generally quite low compared with that of *S. kurzii* and eggplant (Table 5). There was no tendency for pollen fertility to increase with succeeding backcross generations.

3.3. Seed fertility

Plants of the F_1 and all backcross generations, BC_1, BC_2 and BC_3, set fruits after hand-pollination with *S. melongena* pollen. The backcross progenies of the anther-indehiscent-type did not set any fruit without hand-pollination. Fruit set percentage

Table 4. Anther dehiscence in F_1 hybrid plants between *S. kurzii* and *S. melongena* ‘Uttara’, and their backcross generations.

Plant materials	Number of plants	
	Anther dehiscent	Anther indehiscent
F_1	3	0
BC_1	4*	8
BC_2 obtained from an anther-dehiscent BC_1	11*	0
BC_2 obtained from an anther-indehiscent BC_1	1*	10
BC_3 obtained from an anther-dehiscent BC_2	2*	8
BC_3 obtained from an anther-indehiscent BC_2	0	10

Anthers of the parental *S. kurzii* and *S. melongena* ‘Uttara’ dehisced and released large quantities of pollen.

* Some anthers dehisced slightly and released a little pollen.

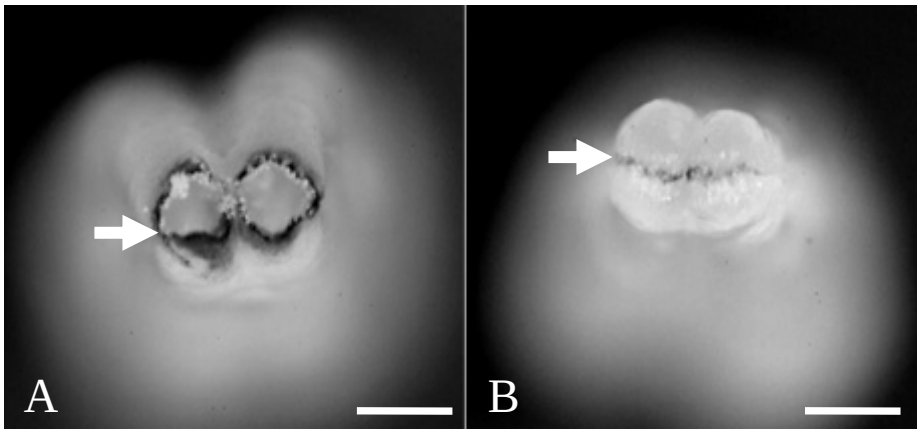

Fig. 8. Anther tips in (A) *S. melongena* ‘Uttara’ and (B) a BC_3 plant. Scale bar = 0.5 mm. Arrows indicate the pore portion of anthers.

Table 5. Stainability and germination ability of pollen, fruit set, and number of seeds per fruit in F_1 hybrid plant between *S. kurzii* and *S. melongena* 'Uttara', and their backcross progenies.

Plant materials	Anther dehiscent (+) or indehiscent (-)	Pollen stainability (%)	Pollen germination ability (%)	Fruit set (%)	Number of seeds per fruit
Solanum kurzii	+	85	82	100	18
S. melongena 'Uttara'	+	96	89	100	351
F_1	+	30	1	50	43
BC_1 Plant 1	+*	55	4	60	74
Plant 2	+*	73	15	80	232
Plant 3	-	81	7	70	142
Plant 4	-	44	4	80	152
Mean		63.3 ± 9.7	7.5 ± 3.0	72.5 ± 5.5	150 ± 37.4
BC_2 Plant 1	+*	62	24	90	440
Plant 2	+*	73	15	100	269
Plant 3	-	64	2	80	295
Plant 4	-	74	15	90	145
Mean		68.3 ± 3.5	14 ± 5.2	90 ± 4.7	287.3 ± 69.9
BC_3 Plant 1	+*	76	49	100	172
Plant 2	+*	71	12	90	375
Plant 3	-	49	13	90	331
Plant 4	-	68	20	90	322
Mean		66 ± 6.8	23.5 ± 10.0	92.5 ± 2.9	300 ± 51.1

* Some anthers dehisced slightly and released a little pollen.

was high in most plants of all generations examined (Table 5). After hand-pollination, all fruits of the BC_3 plants contained seeds. The number of seeds per fruit increased gradually with succeeding backcross generations (Table 5). The average of number of seeds per fruit in BC_2 and BC_3 plants was almost the same as that of the parental *S. melongena*. Mean value of germination rates of seeds from four anther-indehiscent type BC_3 plants was 92%, and that of the parental *S. melongena* was 91%.

3.4. Observation of chromosome associations

Solanum kurzii and eggplant exhibited 12 bivalent (12II) formation at meiotic MI in all the PMCs observed. Also, the pollen non-release BC_3 plants showed 12II formation in all the PMCs observed (Fig. 9).

3.5. Cytoplasm identification

All backcross progenies displayed restriction patterns identical to those of *S. kurzii* in analyses of both cpDNA and mtDNA (Fig. 10).

4. Discussion

Organelle inheritance in most plants is strictly maternal, although there are some exceptions (Reboud and Zeyl, 1994). In analyses of cpDNA and mtDNA in the present study, all the backcross progenies examined exhibited restriction patterns identical to the nonrecurrent female parent, (the cytoplasm donor) *S. kurzii*, indicating that the chloroplasts and mitochondria of the backcross progenies were those from *S. kurzii*. Therefore, the cytoplasm of *S. kurzii* was shown to be inherited maternally. This confirms that the repeated backcross method is suitable to develop cytoplasm substitution lines of eggplant.

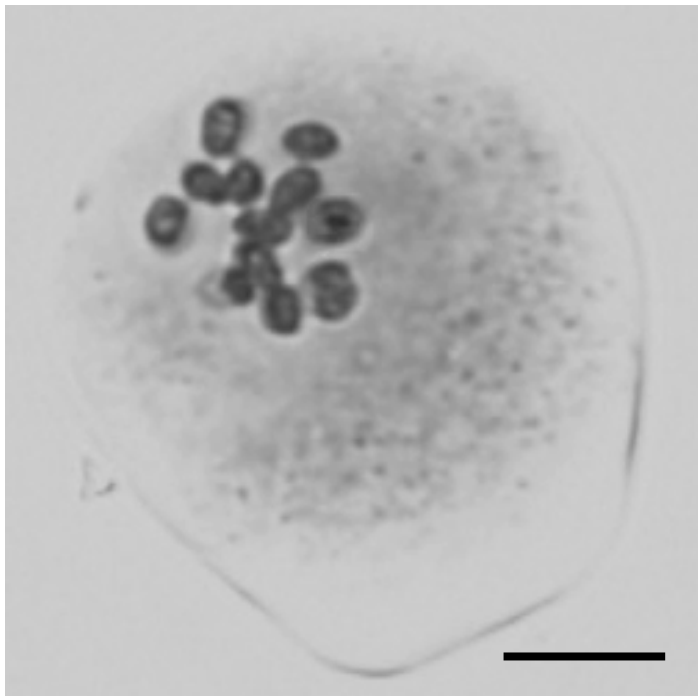

Fig. 9. Meiotic metaphase I in a pollen mother cell of a plant of BC_3 showing 12 bivalent formation. Scale bar = 10 µm.

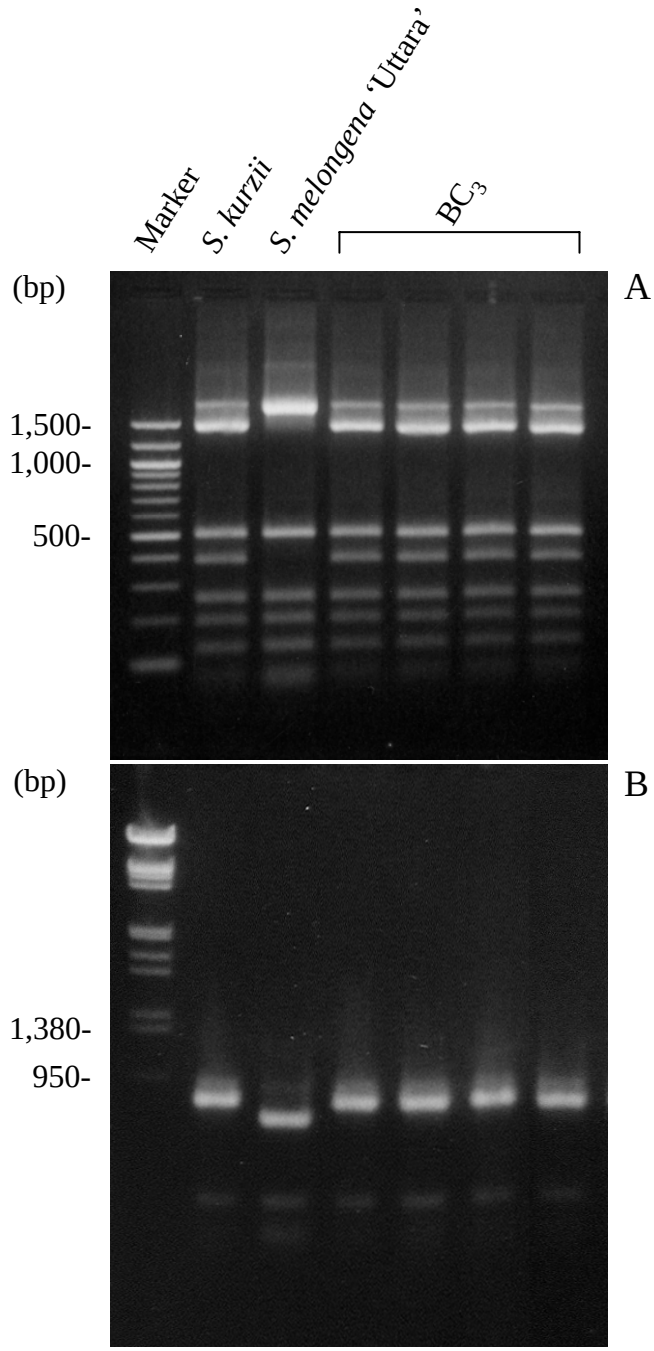

Fig. 10. Restriction enzyme patterns of the (A) *Alu* I digested *rbc*L-ORF106 region of cpDNA and (B) *Scr* FI digested V7 region of the mitochondrial small ribosomal subunit RNA gene of *S. kurzii*, *S. melongena* 'Uttara' and four plants of BC_3.

In cases of true cytoplasmic male-sterility (CMS), the degree of male-sterility is known to increase with each successive backcross generation (McVetty, 1997). In the backcross progenies of the present study, many of the plants of BC_1 and BC_2 exhibited the pollen-release-type, however, in the BC_3, frequency of the anther-indehiscent (pollen-non-release) type plants were high. All the BC_3 plants whose maternal parent was the anther-indehiscent-type were the anther-indehiscent-type without segregation. Therefore this anther indehiscent character is suggested to be a kind of CMS, induced by disharmony between the cytoplasm of *S. kurzii* and the nucleus of *S. melongena* and not to be controlled by a single dominant gene. Although all the backcross progenies produced pollen grains in their anthers, malfunction of the anthers prevented release of these pollen grains.

Pollen fertility of the present F_1 was low. Similar results have been obtained for F_1 hybrids between *S. melongena* and the related *Solanum* species (Rajasekaran, 1970; Rangasamy and Kadambavanasundaram, 1974; Isshiki and Kawajiri, 2002). This low pollen fertility of the F_1 might be attributed to meiotic difficulty arising from hybridity of the nucleus. However, pollen fertility of all the backcross progenies was also generally low and there was no tendency for the fertility to increase with succeeding backcross generations. Complete 12II formation at meiotic MI, as observed in the BC_3 plants examined, also suggests that the low pollen fertility in BC_3 plants could not be attributed to problems at meiosis. These findings suggest that the low pollen fertility seen in the cytoplasm substitution lines was affected by the cytoplasm of *S. kurzii*.

Male-sterility in eggplant has been described in several reports. Functional male-sterility by nuclear genes was reported by Jasmin (1954), Nuttall (1963), Phatak and Jaworski (1989), Phatak *et al.* (1991), and Isshiki and Kawajiri (2002). Chauhan (1984) reported that genetic male-sterility was caused by malfunctioning of the

tapetum. Fang *et al.* (1985) developed cytoplasmic male-sterile lines of eggplant using *S. gilo* (i.e., *S. aethiopicum* Gilo Group) cytoplasm. Functional male-sterile lines were developed using the cytoplasm *S. violaceum* Ort. (*S. indicum* auct. non L.) in our previous study (Isshiki and Kawajiri, 2002). In the present study, the functional male-sterile lines of the anther-indehiscent-type could be induced using the cytoplasm of *S. kurzii*. The present male-sterile lines are similar to our previously developed, functional male-sterile lines using the cytoplasm of *S. violaceum* (Isshiki and Kawajiri, 2002).

Some cytoplasmic male-sterile systems have been reported to be temperature unstable and not to be commercially usable for hybrid seed production (McVetty, 1997). Phatak *et al.* (1991) reported that functional male-sterility of the pollen non-release-type in their eggplant was influenced by the environment and by insects. However, during >4 months of observation, the anther-indehiscent character of our cytoplasm substitution line of eggplant (BC_3 plants) showed stable expression. Therefore, the present male-sterile lines appear promising.

On the other hand, seed fertility was generally high in all the backcross progenies examined. Fruit set percentage of them was high throughout all the generations. There was a tendency that the number of seeds per fruit increased with succeeding generations. The number of seeds per fruit in the BC_3 was also the almost same of the parental *S. melongena*. Furthermore, the mean germination rate of the seeds from four BC_3 plants was the same (92%) of that of the *S. melongena* parent. These findings indicate that cytoplasm of *S. kurzii* has no significant negative effect on seed fertility of eggplant. The present male-sterile lines would be excellent one.

Cytoplasmic male-sterile systems have traditionally been characterised by the restorer genes required to overcome the CMS (McVetty, 1997). In the present study,

however, the restorer genes of our system could not be discovered. Further studies for detecting the restorer genes are essential both for characterization of the system and for recovery of male fertility.

Recently, excellent parthenocarpic lines of eggplant have been developed by genetic engineering (Rotino et al., 1997) and conventional cross-breeding (Kikuchi et al., 2008). If these parthenocarpies can be introduced to our cytoplasmic male-sterile lines, our lines might be utilized for seedless fruit production without hormone treatment.

The present study demonstrates that it is possible to develop a new male sterile line of eggplant by utilizing the cytoplasm of *S. kurzii*.

CHAPTER IV

Development of male sterile line of eggplant utilizing the cytoplasm of *S. virginianum* and their fertility studies

1. Introduction

Solanum virginianum L. (= *Solanum xanthocarpum* Schrad. and Wendl. = *Solanum surattense* Burm.f.) (Fig. 11) belongs to the same subgenus *Leptostemonum*, section *Melongena* of eggplant but have different series (*Sodomela*) than eggplant (*Incaniformia*). Serological and crossability studies (Pearce and Lester, 1979) have shown that *S. virginianum* is less closely related to egg-plant complex. *Solanum virginianum* appear to be closely related to *S. melongena* both on morphological (Bhaduri, 1951) and cytological (Rajasekaran, 1969). Studies in isozymes (Isshiki et al., 1994), cpDNA (Isshiki et al., 1998), mtDNA (Isshiki et al., 2003) and ISSR analysis (Isshiki et al., 2008), *S. virginianum* showed a quite distant relationship with *S. melongena* indicating that taxonomic position of this species should be outside of the section *Melongena*. It is a sympatric weed of the wild ancestor *S. melongena* var. *insanum* from which cultivars of *S. melongena* have been derived (Lester and Hasan, 1991). This species has been reported for its resistance to *Pseudomonas solanacearum* (Hébert, 1985). This species also possesses medical properties, and is widely used to treat respiratory diseases in Indian traditional medicine (Govindan et al., 1999; Govidan et al., 2004). While Sarvayya (1936) was able to produce partial sterility of hybrids between these two species, Rajasekaran (1971) reported the hybrid was completely sterile. However, the cytoplasm of *S. virginianum* has not been utilized for the development of male sterile line of eggplant yet. In this study, we utilized the

Fig. 11. *S. virginianum.*

cytoplasm of *S. virginianum* to develop a new male sterile line of eggplant by continuous backcross method. Backcross progenies were examined for their pollen release ability, pollen fertility, seed fertility, meiosis and organelle DNAs.

2. Materials and methods

2.1. Plant material

To develop a cytoplasmic substitution line of eggplant, an interspecific F_1 hybrid (*S. virginianum* × *S. melongena* 'Senryo Nigou') was continuously backcrossed to *S. melongena* 'Uttara' using 'Uttara' as a recurrent pollen parent. Four backcross generations, BC_1, BC_2, BC_3 and BC_4 were produced.

2.2. Pollen release ability

Anther tips were observed under stereomicroscope for assessing pollen release ability. Anthers from 15 flowers were examined for each plant.

2.3. Pollen fertility

Pollen stainability and *in vitro* germination ability of pollen were investigated for assessing pollen fertility. Pollen grains were stained with acetic carmine and examined under the microscope to determine pollen stainability. *In vitro* germination ability of pollen was investigated by using a germination medium consisted of 1% agar, 5% sucrose and 50 mg/l boric acid. Pollen germination ability was determined after incubation at 25 ºC for four hours. At least 500 pollen grains from each of 5 flowers per plant were assessed for pollen fertility. One BC_1, 11 BC_2, 22 BC_3 and 11 BC_4 plants were examined.

2.4. Seed fertility

Fruit set, number of seeds per fruit and seed germination were examined for assessing seed fertility. The F_1 hybrid and the backcross plants were pollinated with the pollen of *S. melongena* 'Uttara'. The *S. virginianum* and *S.melongena* 'Uttara' were selfed. For seed germination, seeds were sown in soil in a glass house with controlled minimum and maximum temperatures of approximately 15 ºC and 30 ºC, respectively. Seed germination was recorded 30 days after sowing.

2.5. Meiotic observation

Chromosome pairing at meiotic metaphase I (MI) in 30 pollen mother cells (PMCs) from fresh anthers of *S. virginianum*, *S.melongena* 'Uttara', F_1 hybrid and each of the three plants of BC_4 progenies were observed by the smear method with acetocarmine.

2.6. Analysis of organelle DNAs

Chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) were analyzed in *S. virginianum*, *S. melongena* 'Uttara', F_1 hybrid, BC_1 and BC_4 progenies to identify their organelle inheritance. Total DNA was isolated from fresh leaves using the CTAB method described by Murray and Thompson (1980). The cpDNA was analyzed by RFLP analysis of a PCR amplified region between *rbc*L and ORF106 following the method described by Isshiki et al. (1998). The PCR products of cpDNA were digested with *Rsa*I, *Sty*I and *Alu*I restriction enzymes. The mtDNA was analyzed by RFLP analysis of a PCR amplified *nad*7 / 3–4 region (Dumolin-Lapegue et al., 1997) and V7 region of mitochondrial small ribosomal subunit RNA (srRNA) gene (Buiteveld et al., 1998). The amplified fragments were digested with *Alu*I for *nad*7 / 3–4 region and *Scr*FI for V7 region of mitochondrial srRNA gene. The DNA restriction fragments

were separated on a 1.5% agarose gel containing ethidium bromide and were photographed under UV illumination.

3. Results

3.1. Pollen release ability

Solanum virginianum, *S. melongena* 'Uttara' and F_1 hybrid were anther dehiscent, i.e. anthers opened to release pollen (Table 6, Fig. 12). All the plants in backcross generations were anther indehiscent, i.e. anthers did not open to release pollen.

3.2. Pollen fertility

Pollen stainability and germination ability in *S. virginianum* and *S. melongena* 'Uttara' were very high (Table 7, Fig. 13). Pollen fertility of the F_1 hybrid was very low. Pollen stainability and germination ability of the backcross progenies were lower compared with *S. virginianum* and *S. melongena* 'Uttara'. There was no tendency for the pollen fertility to recover with the succeeding backcross generations.

3.3. Seed fertility

The F_1 hybrid and all the backcross progenies observed set fruits with viable seeds when pollinated with the pollen of *S. melongena* 'Uttara'. Seed fertility of the backcross progenies was generally high (Table 8). Fruit set percentage and number of seeds per fruit increased gradually with the succeeding backcross generations. Fruit set percentage of the examined plants in all the backcross generations was high. The number of seeds per fruit of the BC_4 plants was similar to that of the number of seeds obtained from *S. melongena* 'Uttara'. Seed germination rate of the backcross plants was high.

Table 6. Anther dehiscence in F_1 between *S. virginianum* and *S. melongena* 'Senryo Nigou' and four backcross generations.

Anther	Number of plants				
	F_1	BC_1	BC_2	BC_3	BC_4
Dehiscent	1	0	0	0	0
Indehiscent	0	1	11	23	11

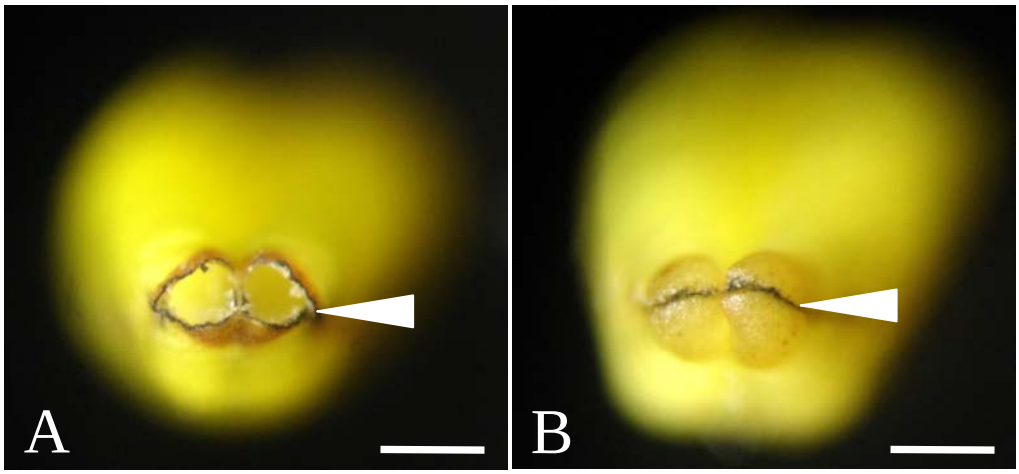

Fig. 12. Anther tips at flowering of (A) *S. melongena* 'Uttara' and (B) a plant of BC_4. Scale bar = 0.5 mm. Arrows indicate the pore portion of anthers.

Table 7. Pollen fertility in *S. virginianum*, *S. melongena* 'Uttara', F_1 hybrid and four backcross generations.

Plant material	Pollen stainability (%)	Pollen germination ability (%)
S. virginianum	97.4	94.5
S. melongena 'Uttara'	95.5	85.5
F_1 hybrid	5.2	5.2
BC_1	66.7	21.7
BC_2 [a]	56.6 ± 4.1	9.2 ± 1.6
BC_3 [a]	41.2 ± 2.6	22.5 ± 2.1
BC_4 [a]	36.6 ± 4.1	11.8 ± 2.9

[a] Data represent mean values with SE of progenies in the backcross generation.

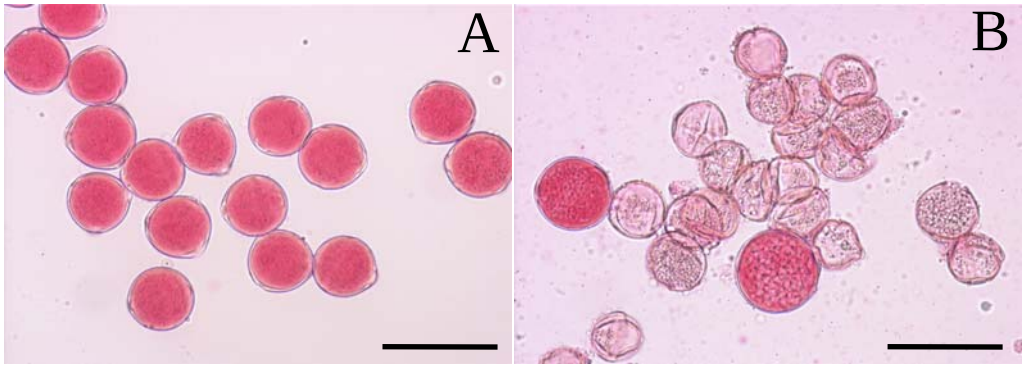

Fig. 13. Acetocarmine stained pollen grains of (A) *S. melongena* 'Uttara' and (B) a plant of BC_4. Scale bar = 50 µm.

Table 8. Seed fertility in *S. virginianum*, *S. melongena* 'Uttara', F_1 hybrid and four backcross generations.

Plant material	No. of cross	Fruit set (%)	No. of seeds per fruit	No. of seeds sown	Seed germination (%)
S. virginianum	12	100	272	100	80
S. melongena 'Uttara'	12	100	532	100	91
F_1 hybrid	26	31	4	40	43
BC_1	11	82	238	50	94
BC_2					
1	7	86	357	100	83
2	13	85	347	-[a]	-
BC_3					
1	14	93	409	100	84
2	11	100	314	-	-
3	15	93	504	-	-
BC_4					
1	12	100	574	100	98
2	10	90	548	-	-
3	10	100	428	-	-

[a] Not investigated.

3.4. Meiotic observation

Complete 12 bivalent formation was observed in all PMCs at meiotic MI in *S. virginianum* and *S. melongena* 'Uttara'. The average chromosome pairing of the F_1 hybrid was 11.7II + 0.6I. Whereas, meiotic behaviour of the BC_4 plants was almost normal (Fig. 14). One of the three BC_4 plants examined showed complete pairing of the chromosomes into 12 bivalents in all PMCs and the average chromosome pairing of the rest two was 11.9II + 0.1I. After meiosis, the microspores were released from the tetrads and after that many microspores degenerated. Due to the degeneration of microspores, many empty pollen grains appeared in the anthers (data not shown).

3.5. Analysis of organelle DNAs

From the analysis of mtDNA, it was found that the restriction patterns of F_1 hybrid, BC_1 and BC_4 plants were identical to that of *S. virginianum* (Fig. 15). From the analysis of cpDNA, it was found that the restriction patterns of F_1 hybrid were identical to those of the *S. virginianum* (Fig. 16). Whereas, three types of restriction pattern were observed in the examined BC_1 plant. The restriction enzyme *Rsa*I digested PCR products showed identical restriction pattern to that of *S. virginianum*. The restriction enzyme *Sty*I digested PCR products showed identical restriction pattern to that of *S. melongena* 'Uttara'. While a nonparental band was observed in addition to parent specific bands from both parents when the PCR products were digested with the restriction enzyme *Alu*I. The following backcross progenies showed the identical restriction patterns as observed in the BC_1 plant.

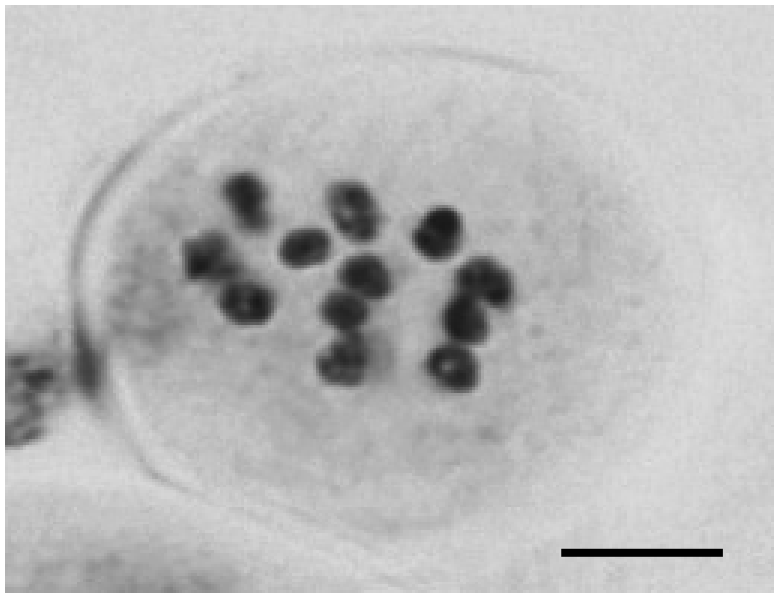

Fig. 14. Meiotic metaphase I in a pollen mother cell of a plant of BC_4 showing 12 bivalents. Scale bar = 10 µm.

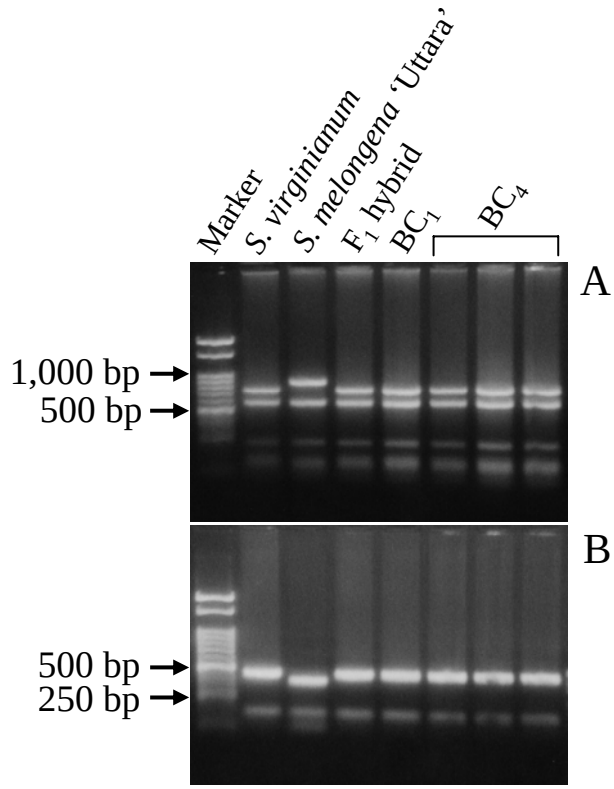

Fig. 15. Restriction enzyme patterns of the (A) *Alu*I digested *nad*7 / 3–4 region and (B) *Scr*FI digested V7 region of mitochondrial small ribosomal subunit RNA gene of *S. virginianum*, *S. melongena* 'Uttara', F_1 hybrid, BC_1 and BC_4 plants.

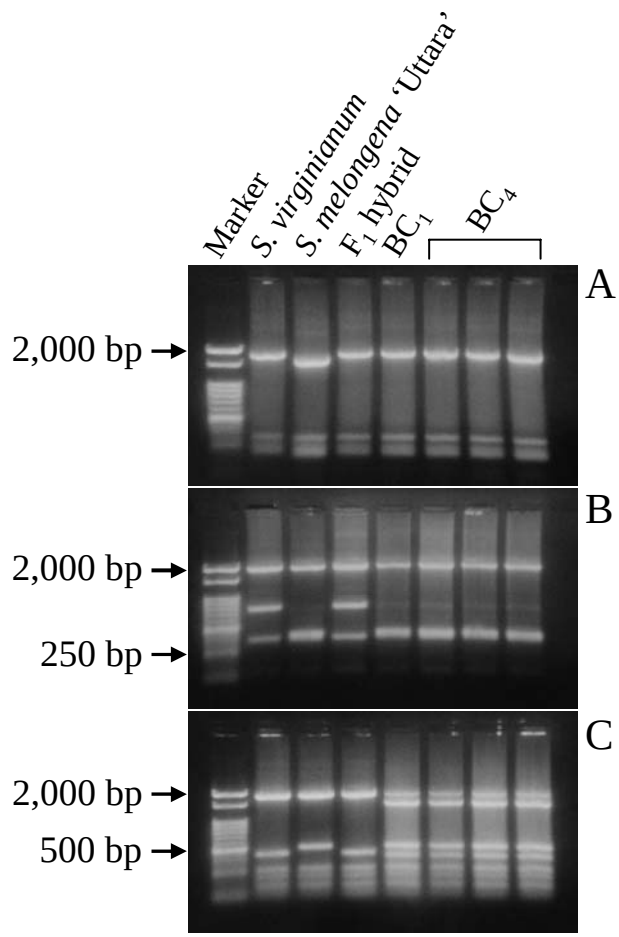

Fig. 16. Restriction patterns of the (A) *Rsa*I (B) *Sty*I and (C) *Alu*I digested *rbc*L-ORF106 region of cpDNA of *S. virginianum*, *S. melongena* 'Uttara', F_1 hybrid, BC_1 and BC_4 plants.

4. Discussion

All the plants in backcross generations examined were anther indehiscent without segregation (Table 6). Malfunction of the anthers prevented the release of pollen grains from the backcross progenies although all the progenies contained pollen grains in their anthers (Fig. 12). This anther indehiscent character is indicated to be a functional male sterility induced by disharmony between the cytoplasmic genes of *S. virginianum* and the nuclear genes of *S. melongena*. Cytoplasmic male sterility arises from interspecific or intergeneric crosses has been reviewed by Edwardson (1970).

Pollen fertility of the F_1 hybrid was lower than those of the parental *S. virginianum* and *S. melongena* 'Uttara' (Table 7). Similar results were obtained for the F_1 hybrids between these two species by Rajasekaran (1971). The F_1 hybrids between *S. melongena* and the related *Solanum* species (Rangasamy and Kadambavanasundaram, 1974; Nishio et al., 1984) were also in accordance with our result. Defective chromosome association at meiotic MI and small segmental differences present in the chromosomes of *S. virginianum* and *S. melongena* might have contributed to lower the pollen fertility of the present F_1 hybrid as reported in the past (Rajasekaran, 1970, 1971).

Pollen fertility of the backcross progenies was also quite lower than those of *S. virginianum* and *S. melongena* 'Uttara' and there was no tendency for the pollen fertility to recover with the following backcross generations (Table 7). Almost normal meiosis in the examined BC_4 plants (Fig. 14) suggested that this low pollen fertility of the backcross progenies was indicated not to be due to meiotic complexity but the effect of the cytoplasm of *S. virginianum.* Similar finding was also observed by Isshiki and Kawajiri (2002) where the cytoplasmic male sterile line of eggplant was developed with the cytoplasm of *S. violaceum*. In order to clarify the specific cause of

the low pollen fertility, further studies should be carried out by analyzing post meiotic processes in the backcross progenies.

Seed fertility of the backcross progenies was usually high (Table 8). The number of seeds per fruit of the BC_4 plants was similar to that of *S. melongena* 'Uttara'. Fruit setting and seed germination rate were also comparable to *S. melongena* 'Uttara'. These results demonstrate that the cytoplasm of *S. virginianum* did not cause significant negative effect on the seed fertility of eggplant.

In most angiosperms, both plastids and mitochondria are maternally inherited (Corriveau and Coleman, 1988). Maternal inheritance of mtDNA of this study is in accordance with most angiosperms as described above, however, different inheritance pattern of cpDNA was observed in the present study. In the examined single BC_1 plant, the paternal and novel restriction patterns were recognized in addition to the maternal restriction one by using different restriction enzymes in PCR-RFLP analysis (Fig. 16). This result suggested that the cpDNA of the BC_1 plant was a recombinant one, i.e. cpDNA was inherited biparentally during backcrossing the F_1 hybrid with the pollen of *S. melongena* 'Uttara. In *Solanum* generally, the generative cell contains a few plastids immediately after the first haploid mitosis but the plastids disappear during the maturation of the generative cell before fertilization (Clauhs and Grun, 1977). In the BC_1 plants of the present study, however, the cpDNA inherited from both parents. This indicates that the cpDNA was present in sperm cell of the *S. melongena* 'Uttara' without disappearance and it was transmitted to the fertilized egg cell during the fertilization process in the ovule of the F_1 hybrid. As all the BC_2, BC_3 and BC_4 progenies showed the same recombinant cpDNA patterns of the BC_1 plant, the recombinant cpDNA might be stable and harmonize with the nuclear genome of *S. melongena*.

All the present backcross progenies, which carried the recombinant cpDNA of the parents and the maternal mtDNA, expressed CMS. Cytoplasmic male sterility (CMS) is usually results from the interaction between nuclear factor and cytoplasmic one, which is mitochondrial one in most cases (Schnable and Wise, 1998), although there are some exceptions (Frankel et al., 1979; Van der Hulst et al., 2004; Ruiz and Daniell, 2005). The present CMS might have occurred by the disharmony between mitochondrial genes of *S. virginianum* and nuclear genes of *S. melongena*, however, it cannot be confirmed now because no backcross progeny which possesses non recombinant cpDNA could be obtained in the present study.

Occurrence of cpDNA recombination event caused by biparental transmition of cpDNA in sexual crossing recognized in the present study is a very rare one and the first evidence in non-tuberous *Solanum* to our knowledge. This finding would be valuable for elucidating diversification of chloroplast in the evolutionary study, although further studies should be needed to reveal the detail mechanism of this event.

In eggplant, functional male sterility of genic male sterility (GMS) has been reported by Jasmin (1954), Nuttall (1963), Chauhan (1984), Phatak and Jaworski (1989) and Phatak et al. (1991). Functional male sterility of CMS having the cytoplasm of *S. violaceum* (Isshiki and Kawajiri, 2002) and the petaloid and vestigial anther type of CMS having the cytoplasm of *S. gilo* Raddi (Fang et al., 1985) have been reported. In this study, an additional functional male sterility (anther indehiscent type) in eggplant could be induced using the cytoplasm of *S. virginianum*. Functional male sterility of eggplant has been reported to be influenced sometimes by the environment and insects (Phatak et al., 1991), however, the present male sterility showed stable expression in our personal observation for more than four months (minimum and maximum temperatures for that period were 26 ºC and 38 ºC,

respectively), which justify the stability of the present CMS line. To be useful for breeding purpose, a CMS system should fulfill some requirements, such as stable expression of male sterility with normal seed fertility. The present CMS possesses these traits.

CMS systems have traditionally been characterized by the restorer genes required to overcome the CMS and to provide male fertile progeny in the male sterile cytoplasm (McVetty, 1997). As the fertility restorer gene of the present CMS system could not be discovered yet, further research would be imperative to find it. On the other hand, parthenocarpic eggplant lines have been developed by genetic engineering (Rotino et al., 1997; Donzella et al., 2000) and conventional cross-breeding (Kikuchi et al., 2008) recently. Incorporating these parthenocarpic characters to the present CMS system will be very useful for producing high quality seedless fruits and for fruiting of hybrid cultivars without discovering the fertility restorer genes or treating phytohormone.

The present study clarified that the cytoplasm of *S. virginianum* is available for producing male sterile line of eggplant. In addition, this result contributes to expand the male sterility source of eggplant.

CHAPTER V

Anther culture for producing pure lines of *S. virginianum* induced male sterile line of eggplant

1. Introduction

In a previous study, a cytoplasmic male sterile (CMS) line of eggplant (*Solanum melongena* L.) was obtained utilizing the cytoplasm of *S. virginianum* by continuous backcrossing (Khan and Isshiki, 2008). An interspecific F_1 hybrid between *S. virginianum* and eggplant was continuously backcrossed to eggplant by using *S. virginianum* as cytoplasm donor and eggplant as a nucleus donor and four backcross progenies, BC_1, BC_2, BC_3 and BC_4 were produced. The backcross progenies showed anther indehiscent type of functional male sterility. All the backcross progenies produced pollen grains in their anthers but malfunction of the anthers prevented release of these pollen grains. This CMS line has high seed fertility. Therefore, this is a useful CMS line of eggplant. For breeding purpose, homozygous plants are needed. The conventional system of producing hybrid seeds through cytoplasmic male sterility is a time-consuming process that involves several years of repeated self-pollination and selection to develop the parental lines. In several species, this procedure is impeded by the necessity of creating CMS analogues through repeated backcrossing of the parental lines to the CMS donor to develop the CMS components (Welsh, 1981). Anther culture technique is a useful means for haploid and homozygous diploid production from microspores and saves time in obtaining pure lines. Further, there is no report in developing pure lines of CMS line of eggplant in our knowledge. Keeping this objective in mind, the purpose of the present study is to produce pure lines of *S. virginianum* induced male sterile line of eggplant by anther culture.

2. Materials and methods

2.1. Material

Anther of a plant of BC_4 (CMS line) developed by continuous backcrossing of an interspecific F_1 hybrid (*S. virginianum* × *S. melongena* 'Senryo Nigou') to *S. melongena* 'Uttara' using 'Uttara' as a recurrent pollen parent was used for culture.

2.2. Pollen fertility

Pollen stainability and *in vitro* germination ability of pollen of *S. melongena* 'Uttara' and the BC_4 plant were investigated for assessing pollen fertility. Pollen grains from freshly opened flowers were extracted from the anthers by dissection, then smeared in 1% acetic carmine to assess their staining ability using the method described by Singh (2002). *In vitro* germination ability of pollen was investigated according to Singh (2002) with a slight modification following germination medium consisted of 1% agar, 5% sucrose and 50 mg/l boric acid. Pollen germination ability was determined after incubation at 25 ºC for four hours. For assessing pollen fertility, more than 500 pollen grains were examined for each plant.

2.3. Anther culture

Anthers containing microspores at unicleate stage were selected for culture. The unopened flower buds were sterilized with 70% ethanol for 10 s and then with 1% sodium hypochlorite solution for 10 min. They were then rinsed 3 times in sterile water. After sterilization, anthers were cultured in a basal medium composed of Murashige and Skoog inorganic salts plus 3% sucrose, 0.2% gelrite, 1% charcoal and supplemented with 0.1 mg/l both of 2,4-D and kinetin. The medium was adjusted to

pH 5.8. The medium containing anthers were heated to 35ºC for first 8 days in dark and then transferred to 25 ºC under a 16 h photoperiod.

2.4. Regeneration of plants

The embryoids derived from anthers were transplanted to basal medium plus 5% sucrose, 0.8% agar and cultured for two month until roots appeared. The regenerated plant transplanted in a plastic pot filled with vermiculite and acclimatized for 1 month in room temperature.

2.5. Ploidy determination

Chromosome number in the root tips was counted for ploidy determination. Roots were cut approximately 1 cm from the tips and pretreated with 0.05% colchicine for 2.5 hours at 20ºC and fixed in the mixture of acetic acid and ethyl alcohol (1 : 3 v/v) before hydrolyzing at 60ºC for 10 minutes in 1N HCL. Then they were stained with leucobasic fuchsine for 20 minutes. Root tips were placed on a glass slide and a drop of 45% acetic acid was added. A cover slip was placed over the root tip and gentle pressure was applied to the cover slip to smear the root tip and observed under microscope.

2.6. Analysis of organelle DNAs

Chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) were analyzed in *S. virginianum*, *S. melongena* 'Uttara', BC_4 and the regenerated plant to identify cpDNA and mtDNA pattern. Total DNA was isolated from fresh leaves using the CTAB method described by Murray and Thompson (1980). The cpDNA was analyzed by RFLP analysis of a PCR amplified region between *rbc*L and ORF106 following the

method described by Isshiki et al. (1998). The PCR products of cpDNA were digested with *Rsa*I, *Sty*I and *Alu*I restriction enzymes. The mtDNA was analyzed by RFLP analysis of a PCR amplified *nad*7 / 3–4 region (Dumolin-Lapegue et al., 1997) and V7 region of mitochondrial small ribosomal subunit RNA (srRNA) gene (Buiteveld et al., 1998). The amplified fragments were digested with *Alu*I for *nad*7 / 3–4 region and *Scr*FI for V7 region of mitochondrial srRNA gene. The DNA restriction fragments were separated on a 1.5% agarose gel containing ethidium bromide and were photographed under UV illumination.

3. Results

3.1. Pollen fertility

Pollen stainability and in vitro germination ability of pollen of *S. melongena* 'Uttara' were very high (Table 9 and Fig. 17). Pollen stainability and germination ability of the BC_4 plant were very low compared with *S. melongena* 'Uttara'.

3.2. Anther culture

From a total of 360 cultured anthers, 7 embroids were formed after 30 days in culture (Fig. 18). From these embroids a single plantlet was regenerated (Fig. 19).

3.3. Ploidy determination

The regenerated plant (Fig. 20) have both haploid ($2n = 12$) and diploid cells ($2n = 24$) in the root tips (Fig. 21).

Table 9. Pollen stainability and pollen germination ability of *S. melongena* 'Uttara' and the BC_4 plant used for anther culture.

Plant material	Pollen stainability (%)	Pollen germination ability (%)
S. melongena 'Uttara'	95.5	85.5
BC_4	18.5	1.9

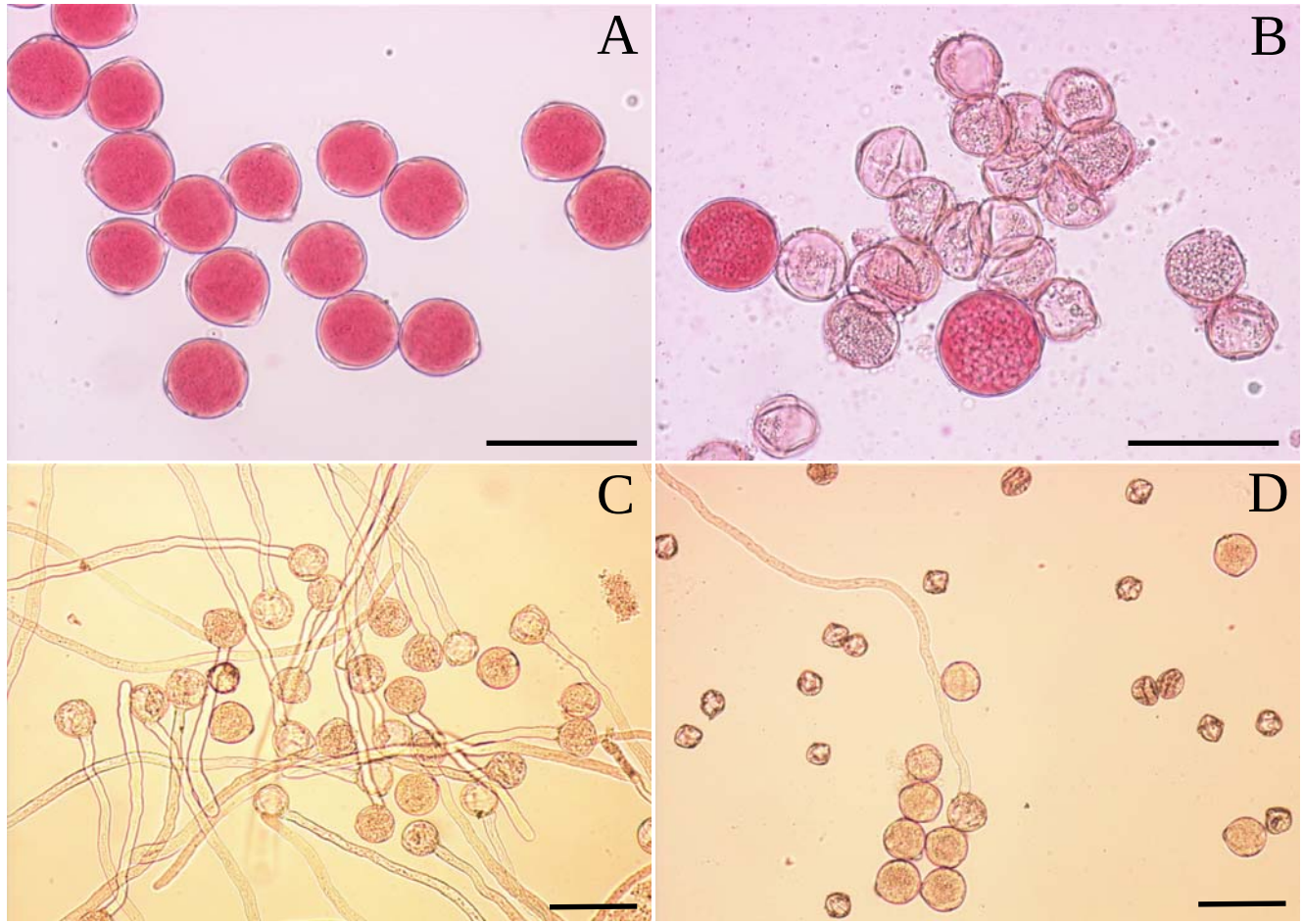

Fig. 17. Acetocarmine stained and *in vitro* germinated pollen grains of *S. melongena* 'Uttara' (A and C) and the BC_4 plant (B and D). Scale bar = 50 µm.

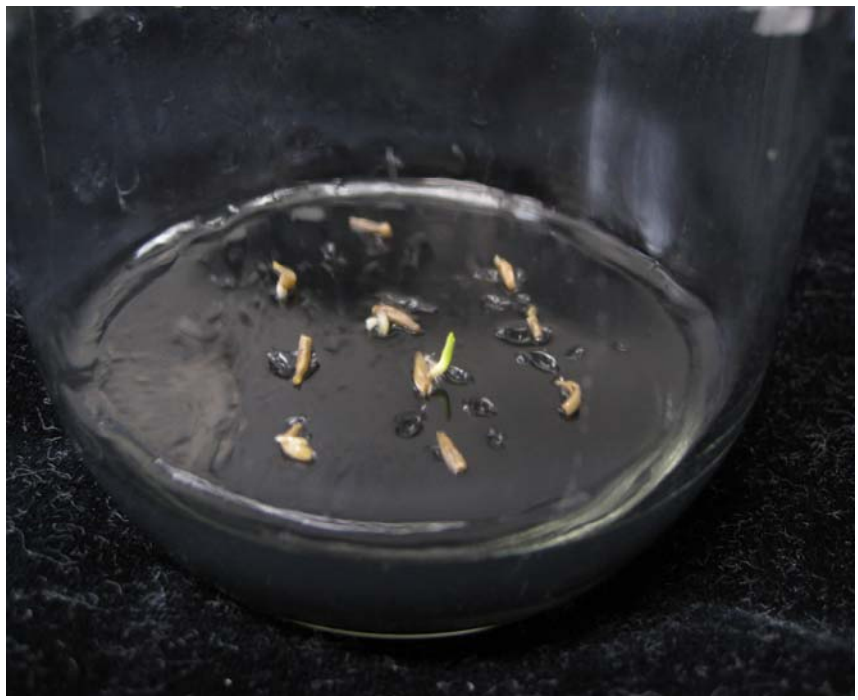

Fig. 18. Embryoid formation from anther of the CMS line (BC_4). White arrows indicate the embryoids.

Fig. 19. Plantlet formation from anther of the CMS line (BC_4).

Fig. 20. Regenerated plant from anther of the CMS line (BC_4).

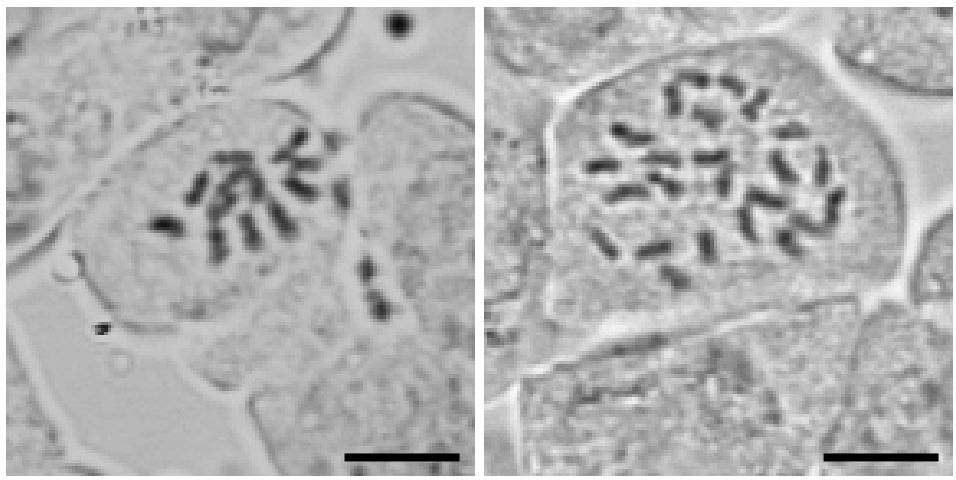

Fig. 21. Somatic chromosomes of haploid cell (2*n* = 12) (left), and diploid cell (2*n* = 24) (right) in root tips of a plantlet regenerated from anther culture of the CMS line of eggplant. Scale bar = 10 μm.

3.4. Analysis of organelle DNAs

PCR-RFLP analysis of cpDNA and mtDNA of the regenerated plant revealed that regenerated plant had the same patterns of cpDNA (Fig. 22) and mtDNA (Fig. 23) as in its mother plant (BC_4 plant used for anther culture).

4. Discussion

Several researchers have reported on the production of haploid plants from anthers of eggplant (Dumas De Vaulx and Chambonnet, 1982; Isouard et al., 1979; Matsubara et al., 1992). But there is no report on anther culture for producing pure lines of a male sterile line of eggplant. In this study, we obtained a single regenerated plant from 360 cultured anthers. Rotino et al. (1987) and Tuberosa et al. (1987) obtained haploid plants in eggplant at the rates of 4.4 and 4.8%, respectively. The frequency of regenerated plants from anther in this study was very low compared to eggplant. One of the reason of obtaining regenerated plant in low frequency may be due to very low pollen fertility of the BC_4 plant compared to eggplant. Another reason may be the influence of genotype on anther culture response. Haploid plant regeneration in eggplant is influenced by genotype, temperature, culture conditions, growth regulators and anther stage (Rotino et al. 1987; Rotino, 1996).

Spontaneous chromosome doubling of haploids was observed in several species (Hamaoka et al., 1991; Miyoshi, 1996). The regenerated plant of the present study have both haploid ($2n = 12$) and diploid cells ($2n = 24$) in the root tips (Fig. 20). Coexistence of haploid and diploid cells in root tips indicates that the root tips were chimeric and supports the view that the regenerated plant had been originally a haploid.

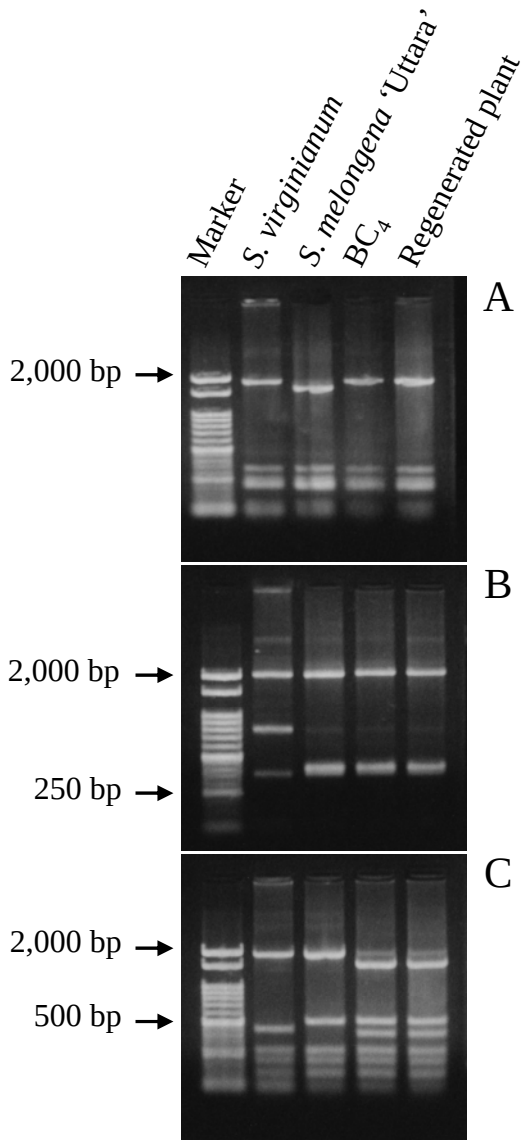

Fig 22. Restriction enzyme patterns of the (A) *Rsa*I (B) *Sty*I and (C) *Alu*I digested *rbc*L-ORF106 region of cpDNA in *S. virginianum, S. melongena* 'Uttara', BC_4 and the regenerated plant.

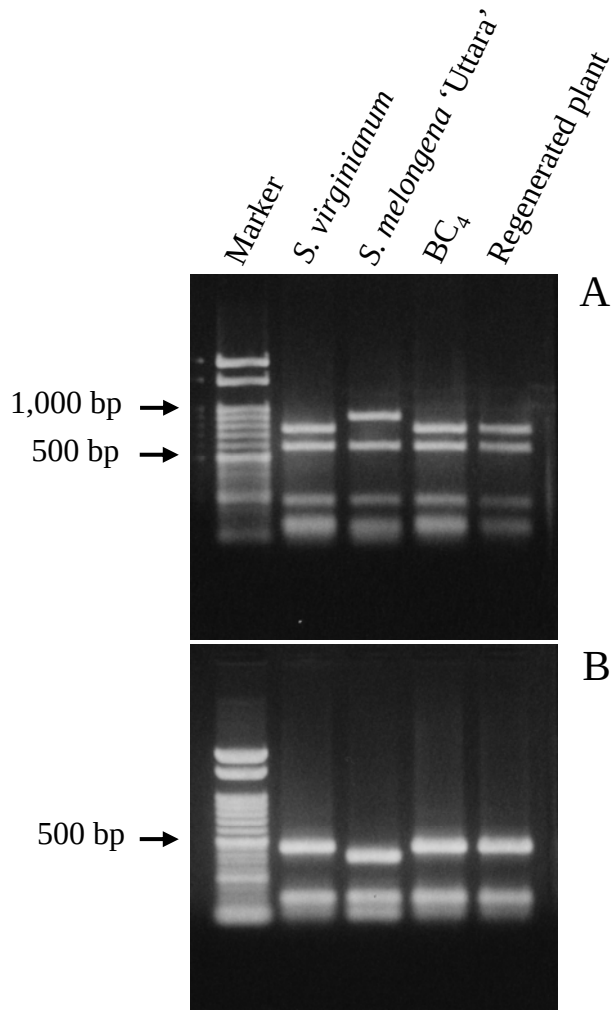

Fig. 23. Restriction patterns of the (A) *Alu*I digested *nad*7 / 3–4 region and (B) *Scr*FI digested V7 region of mitochondrial small ribosomal subunit RNA gene in *S. virginianum*, *S. melongena* 'Uttara', BC_4 and the regenerated plant.

PCR-RFLP analysis of cpDNA and mtDNA of the regenerated plant revealed that regenerated plant had the same patterns of cpDNA and mtDNA as in its mother plant (BC_4 plant used for anther culture), indicating that the regenerated plant preserved the cpDNA and mtDNA from its mother plant. These results indicate that the regenerated plant originated from the microspore and chromosome doubling of the regenerated plant may go on spontaneously. The present study demonstrates that it is possible to develop purelines of a CMS line of eggplant with the cytoplasm of *S. virginianum* by anther culture although the pollen fertility was low.

CHAPTER VI

General discussion

In this study, the possibility of developing male sterile lines of eggplant (*Solanum melongena* L.) was investigated utilizing the cytoplasms of wild *Solanum* species. The cytoplasms of *S. anguivi* and *S. kurzii* in section *Oliganthes* and *S. virginianum* in section *Melongena* were utilized to develop cytoplasm substitution lines of eggplant by continuous backcross method. Maternal inheritance of cpDNA and mtDNA of the *S. anguivi* and *S. kurzii* induced CMS lines indicates that the cytoplasms of *S. anguivi* and *S. kurzii* were successfully substituted for that of eggplant. However, backcross progenies of *S. virginianum* induced CMS line carried recombinant cpDNA of the parents and maternal mtDNA. In the present study, pollen non-formation and anther indehiscent type of sterility was induced in eggplant by the cytoplasms of wild *Solanum* species. Pollen non-formation type sterility was induced by the cytoplasm of *S. anguivi* and anther indehiscent type of sterility was induced by the cytoplasms of *S. kurzii* and *S. virginianum*. The anthers of pollen non-formation type plants were completely devoid of pollen grains and no meiosis was detected. Whereas, malfunction of the anthers prevented the release of pollen grains of anther indehiscent type male sterile plants although they contained pollen grains in their anthers. The present CMS was shown to be attributed to incompatibility between the cytoplasms of wild *Solanum* species and nuclear genes of eggplant. Pollen fertility, which was assessed from pollen stainability and *in vitro* germination ability of pollen in this study, were found lower in the backcross progenies than that of eggplant. Pollen stainability and *in vitro* germination ability found in the backcross progenies (BC_3) between *S. kurzii* and eggplant were 66% and 23.5% respectively. Whereas, pollen

stainability and *in vitro* germination ability found in the backcross progenies (BC_4) between *S. virginianum* and eggplant were 36.6% and 11.8% respectively. These results indicate that cytoplasms of wild *Solanum* species used in this study, strongly influenced the anther morphology, pollen formation ability, pollen release ability and pollen fertility in eggplant and induced cytoplasmic male sterility. Isshiki and Kawajiri, (2002) reported functional male sterility of CMS having the cytoplasm of *S. violaceum*. Backcross progenies (BC_4) of this line showed about 25% pollen stainability. Fang et al. (1985) reported petaloid (the anthers have transformed to narrow incurved petals) and vestigial anther (the anthers do not produce any pollen) sterile type CMS. Therefore, the nuclear-cytoplasmic interactions on anther morphology and pollen fertility of *S. melongena* seems to vary with difference of the cytoplasms.

In the present study fruit set percentage, number of seeds per fruit and seed germination rates were investigated to assess seed fertility. Seed fertility found in the backcross progenies of the CMS lines was generally high. There was a tendency that the fruit set percentage, number of seeds per fruit and seed germination rates increase with succeeding generations. This may be due to the genotype of backcross progeny becomes increasingly similar to eggplant by repeated backcrossing. High fruit set percentage and seed germination rates were observed usually in the later backcross progenies of all the CMS lines. Number of seeds per fruit was found high in *S kurzii* and *S. virginianum* induced CMS lines and moderately high in *S. anguivi* induced CMS line. These findings indicate that these cytoplasms had no significant negative effect on seed fertility of eggplant.

Some cytoplasmic male-sterile systems have been reported to be temperature-unstable and not to be commercially usable for hybrid seed production (McVetty,

1997). Phatak et al. (1991) reported that functional male-sterility of the pollen non-release-type in their eggplant was influenced by the environment and by insects. However, during more than 4 months (minimum and maximum temperatures for that period were 26 ºC and 38 ºC, respectively) of observation, the anther indehiscent character of our cytoplasm substitution line of eggplant utilizing the cytoplasm of *S. kurzii* and *S. virginianum* showed stable expression. Male sterile line of eggplant developed by utilizing the cytoplasm of *S. anguivi* showed pollen non-formation type male sterility and there is no risk of pollen release from anthers, resulting in stable expression. Therefore, these three CMS lines appear promising and would be effectively utilized for the production of F_1 hybrid seeds and seedless fruits of eggplant.

Backcross progenies of *S. kurzii* induced CMS line showed segregation of anther dehiscent and indehiscent types. But all the plants in backcross progenies of *S. virginianum* induced CMS line showed anther indehiscent without segregation. This indicates the presence of higher incompatibility between the cytoplasm of *S. virginianum* and nuclei of eggplant than the cytoplasm of *S. kurzii* and nuclei of eggplant. The present author also try to substitute the cytoplasm of another wild *Solanum* species, *S. macrocarpon* L., for that of eggplant. If the cytoplasmic substitution of other wild species for eggplant will be successfully accomplished, the substitution lines will be useful to elucidate tendencies of phylogenetic relationship and nuclear-cytoplasmic incompatibility between the species toward male sterility.

Male sterile plants induced by *S kurzii* and *S. virginianum* cytoplasms exhibited abnormality in microsporogenesis. Degeneration of microspore occurred after releasing from tetrads. Due to the degeneration of microspores, many empty pollen grains appeared in the anthers. Male sterile plants induced by *S. anguivi*

cytoplasm exhibited absence of meiosis and the anthers of no pollen grains. Therefore, different cytoplasms of wild species also had different influence on meiosis of eggplant. Yamashita et al. (1999), reported degeneration of of microspore protoplasms after pollen grain mitosis in pollen sterile B_5 progenies. Grun and Aubertin (1966) observed the cytological expression of male sterility in *Solanum* and classified male-sterile plants into four groups on the basis of their anther contents, namely, (a) A blockage resulted from cessation of development principally during the stages of meiosis of apparently normal sporocytes. This produced anthers in open flowers that usually contained monads or dyads. (2) Disorganization of sporocytes during first meiotic prophase resulted in irregularity of disjunction during the two meiotic divisions. Sporocytes produced quartet-stage clumps having more than four cells. Microspores failed to grow beyond early stages of exine development. (3) Abnormal small pollen having a very thick exine. (4) Normal pollen present in anthers that lacked terminal pores. Male sterility in eggplant was associated with the malfunctioning of the tapetum (Chauhan, 1984). It is necessary to observe the tapetal behaviour during the microsporogenesis of these CMS lines of eggplant. It is interesting to elucidate the expression of genes, which function in anthers of the CMS lines of eggplant obtained in this study. Further study is needed to find out the causes of pollen non-formation in the pollen non-formation type CMS plants induced by *S. anguivi* cytoplasm. In order to clarify the specific cause of the low pollen fertility, further studies should be carried out by analyzing post meiotic processes in the backcross progenies of the CMS lines developed utilizing *S kurzii* and *S. virginianum* cytoplasms.

The fertility restoring gene (*Rf*) which originated in the nuclear genome of *S. anguivi* was discovered in the pollen formation type backcross progenies between *S.*

anguivi and eggplant. In our knowledge, no fertility restoring gene (*Rf*) has been found in the CMS system of eggplant. Therefore, proper characterization of this *Rf* gene is necessary. For exact elucidation of mode of inheritance of the *Rf* gene, genetic analyses using test cross progenies, such as, selfed ones of the pollen formation backcross progenies etc. should be imperative. Fertility restorer gene of the present CMS systems developed utilizing *S. kurzii* and *S. virginianum* cytoplasms could not be discovered yet. Further studies for detecting the restorer genes are essential both for characterization of the system and for recovery of male fertility.

Molecular markers linked to the *Rf* gene should be developed. After successful development of markers it will be possible to apply marker-associated selection (MAS) (Melchinger, 1990) of the male fertile and male sterile plants in the breeding of eggplant possessing the cytoplasm of *S. anguivi*. Recently some of the fertility restoring genes in maize (Cui et al., 1996) and Tabacco (Hernould et al., 1997) have been successfully cloned. Isolation of the *Rf* gene is also necessary for elucidation of the genes controlling pollen fertility.

It is difficult to determine the cytoplasmic gene concerning with the CMS. CMS occurs in a variety of plant species and is often associated with novel mitochondrial open reading frames, which interfere with mitochondrial function and pollen development. CMS systems have been identified and characterized in a variety of plants, including common bean, beet, maize, onion, petunia, rice, rye, sorghum, sunflower, and wheat (Schnable and Wise, 1998). Several CMS systems have been characterized in the *Cruciferae* family: Polima, Napus, Ogura, and Anand CMS are well-characterized CMS systems belonging to the *Brassica* genus and Ogura and Kosena CMS are well-characterized CMS systems belonging to the *Raphanus* genus (Homme and Brown, 1993; Homme et al., 1997; Handa et al., 1995). CMS related

region and gene in eggplant has not been reported yet. Therefore, molecular markers specific to CMS in eggplant is needed to study.

For practical use of the male sterile lines of eggplant in F_1 seed production, the following studies are required.

1) The high level of stability of the CMS is essential for its practical use in F_1 seed production. Some male sterility systems have been reported to be influenced by the environment and insects (Phatak et al., 1991; McVetty, 1997). Photoperiod sensitive CMS was discovered in wheat (Murai and Tsunewaki, 1993; Murai et al., 1995; Ogihara et al., 1997). In chive (*A. schoenoprasum* L.), temperature-sensitive and tetracycline-sensitive CMS plants were studied in detail (Tatlioglu, 1982, 1985, 1986). Although the male sterile lines in this study showed stable expression in our observation, the confirmation of stability of these CMS in different temperatures, photoperiods and environmental conditions is necessary for certification of the practical use of them in F_1 seed production of eggplant.

2) The cytoplasm of *S. anguivi*, *S kurzii* and *S. virginianum* functioned as a male sterile cytoplasm in eggplant cv. 'Uttara'. Transfer of these cytoplasms into other cultivars of eggplant will increase the genotypic diversity, which contributes to the F_1 breeding of eggplant.

3) High combining ability is a desirable factor in breeding of F_1 cultivars of eggplant. It is necessary to conduct the comprehensive tests of combining abilities between various cultivars of eggplant. The utilization of high combining ability in F_1 seed production of eggplant will efficiently improve the quality and quantity of this crop. Breeders can transfer the CMS character found in the present study to their desired eggplant cultivars by using the desired cultivars as a recurrent pollen parent, which can save valuable time.

4) As the fertility restorer gene of the *S kurzii* and *S. virginianum* induced CMS systems could not be discovered yet, further research would be imperative to find it.

5) Parthenocarpic fruits are seedless, a desirable fruit character in eggplant. Seedless fruits of eggplant are generally more appetizing than seeded ones and preferred by consumers. Recently, parthenocarpic eggplant lines have been developed by genetic engineering (Rotino et al., 1997; Donzella et al., 2000) and conventional cross-breeding (Kikuchi et al., 2008). Incorporating these parthenocarpic characters to the present CMS system will be very useful for producing high quality seedless fruits and for fruiting of hybrid cultivars without discovering the fertility restorer genes or treating phytohormone.

6) Anther culture of male sterile lines of eggplant induced by *S. anguivi* and *S. kurzii* cytoplasms is needed to produce purelines. It is presumed that the pollen formation type backcross progenies possessing *S. anguivi* cytoplasm had the genotype *Rfrf*. From anther culture of pollen formation type backcross progenies, we can get haploid plants having *rf* or *Rf* for pollen fertility gene. By chromosome doubling of these plants, we can get pollen non-formation type male sterile plants carrying homozygous recessive fertility non-restoring gene (*rfrf*), as well as pollen formation type male fertile plants carrying homozygous dominant fertility restoring gene (*RfRf*). The doubled haploid male sterile plants could be used as pureline for production of hybrids and male fertile plants could be used as a restorer line for hybrid seed production program after proper characterization of the *Rf* gene.

CHAPTER VII

Summary

Eggplant (*S. melongena* L.) is a widely cultivated and economically important vegetable crop in many countries all over the world. In hybrid seed production of eggplant, the undesirable selfing and the necessity of labor-intensive manual emasculation (removal of anthers) are the two main quandaries. Incorporation of male sterile character in female parent lines could obviate emasculation in the hybridization process thus reducing the time, labor and cost for the hybrid seed production of eggplant. Male sterile character is also useful for seedless fruit production of eggplant. Seedless fruits of eggplant are generally more appetizing than seeded ones and preferred by consumers. It is strongly desired to widen the range of male sterility systems for their practical uses, such as hybrid seed production and seedless fruit production, in eggplant. Therefore, the purpose of this study is to investigate the possibility of developing male sterile lines of eggplant (*Solanum melongena* L.) utilizing the cytoplasms of wild *Solanum* species. The cytoplasms of *S. anguivi* and *S. kurzii* in section *Oliganthes* and *S. virginianum* in section *Melongena* were utilized to develop cytoplasm substitution lines of eggplant by continuous backcross method using eggplant as a nucleus donor and wild species as cytoplasm donor.

The cytoplasm of *S. anguivi* was successfully substituted for that of eggplant. Pollen non-formation type sterility induced in eggplant by the cytoplasm of *S. anguivi*. The anthers of the pollen non-formation type plants were completely devoid of pollen grains and no meiosis was detected. From the segregation of the pollen formation ability of the backcross progenies, it is assumed that a single dominant fertility restoring gene controls the pollen formation of the *S. melongena* with the cytoplasm of

S. anguivi. High fruit set percentage, moderately high number of seeds per fruit and high seed germination rate found in the backcross progenies indicate high seed fertility of this male sterile line. The present results indicate that the cytoplasm of *S. anguivi* is useful for inducing male sterility in eggplant.

The cytoplasm of *S. kurzii* was successfully substituted for that of eggplant. Anther indehiscent type functional male sterility was found in backcross progenies. This anther indehiscent character was induced by disharmony between the cytoplasm of *S. kurzii* and the nucleus of *S. melongena*. Pollen fertility in all backcross progenies was lower than those of the parental species. Seed fertility was generally high in all the backcross progenies examined. The present results indicate that the cytoplasm of *S. kurzii* is useful for inducing male sterility in eggplant.

The cytoplasm of *S. virginianum* was utilized to substitute for that of eggplant by continuous backcross method. Backcross progenies carried recombinant cpDNA of the parents and maternal mtDNA. Anther indehiscent type of male sterility was expressed in backcross progenies. This anther indehiscent character is indicated to be a functional male sterility induced by disharmony between the cytoplasmic genes of *S. virginianum* and the nuclear genes of *S. melongena*. Pollen fertility in all backcross progenies was quite lower than those of the parental species. Seed fertility was high in all the backcross progenies examined. The present results indicate that the cytoplasm of *S. virginianum* is useful for inducing male sterility in eggplant.

Anther culture was performed to produce pure lines of *S. virginianum* induced CMS line of eggplant. From a total of 360 cultured anthers a single plantlet was regenerated. The regenerated plant has both haploid ($2n = 12$) and diploid ($2n = 24$) cells in the root tips which indicate that the regenerated plant was originated from the microspore and chromosome doubling may go on spontaneously. This study revealed

that anther culture is useful to develop pure lines of *S. virginianum* induced CMS line of eggplant.

This study demonstrated that it is possible to develop male sterile lines of eggplant with the cytoplasms of wild *Solanum* species, *S. anguivi*, *S. kurzii* and *S. virginianum* and that anther culture is useful to develop pure lines of *S. virginianum* induced CMS line of eggplant. The male sterile lines of eggplant developed in this study are potentially valuable for simplifying and reducing the time, labor and cost for the production of F_1 hybrid seeds and seedless fruits of eggplant.

要　約

本研究は、ナス属野生種の細胞質を用いてナス（*Solanum melongena*）の雄性不稔系統育成を試みたものである。ナス属野生種の３種、*S. anguivi*、*S. kurzii* および *S. virginianum* の細胞質を用いて戻し交雑による細胞質の置換を行い、稔性の解析を行った。

戻し交雑によってナスの細胞質を *S. anguivi* のもので置換することに成功した。細胞質置換系統において、花粉を形成する個体と花粉を形成しない雄性不稔性を発現する個体が出現し、この遺伝分離の結果から、この雄性不稔性が単一の優性の稔性回復遺伝子が稔性回復に関与する細胞質・核遺伝子型であることが明らかとなった。雄性不稔個体では、花粉母細胞が減数分裂前に崩壊していることが観察され、このことが花粉を形成しない原因であることが明らかになった。雄性不稔個体は、種子稔性が高かったことから、雄性不稔系統としての実用価値が高いと考えられた。

戻し交雑によってナスの細胞質を *S. kurzii* のもので置換することに成功した。この細胞質置換系統はすべて、花粉を生産するものの、開花しても開葯せず花粉を放出しないタイプの機能的雄性不稔性を発現した。また、雄性不稔性の発現に関して遺伝分離が認められなかったことから、この雄性不稔性は細胞質単独型であると考えられた。この雄性不稔系統も種子稔性が高かったことから、実用性が高いと考えられた。

戻し交雑によってナスの細胞質を *S. virginianum* のもので置換することを試みた結果、戻し交雑後代はすべて、ミトコンドリア DNA に関しては置換に

成功したが、葉緑体 DNA はナスと *S. virginianum* の組換え型のものになった。この系統は、上記の *S. kurzii* の細胞質をもつ雄性不稔系統と同様の機能的雄性不稔性を発現した。また、雄性不稔性の発現に関して遺伝分離が認められなかったことから、この雄性不稔性は細胞質単独型であると考えられた。この雄性不稔系統も種子稔性が高かったことから、実用性が高いと考えられた。

Solanum virginianum の細胞質をもつナスの雄性不稔系統の葯培養を行った．その結果，1 個体の小植物体を再生することに成功し、その個体において半数性（2*n*=12）および二倍性（2*n*=24）の細胞が観察された．このことから，再生個体が花粉由来であることが推定され、葯培養で本細胞質雄性不稔系統の純系を作出できることがわかった.

本研究の結果、ナス属野生種の *S. anguivi*、*S. kurzii*、および *S. virginianum* の細胞質を用いて実用性の高いナスの３種類の雄性不稔系統の育成に成功した。さらに、葯培養によって *S. virginianum* の細胞質をもつ雄性不稔系統の純系を作出することが可能であることも明らかにした。

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