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One-step generation of cytoplasmic male sterility by fusion of mitochondrial-inactivated tomato protoplasts with nuclear-inactivated *Solanum* protoplasts

(Lycopersicon esculentum/Solanum acaule/Solanum tuberosum/Solanum lycopersicoides/Nicotiana tabacum)

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ABSTRACT **Mesophyll protoplasts of *Lycopersicon esculentum* were treated with iodoacetamide to inactivate mitochondria, and protoplasts of *Solanum acaule* and *Solanum tuberosum* were irradiated with γ- or x-rays to inactivate nuclei. Mixtures of protoplasts thus modified were treated with Ca^{2+} and polyethylene glycol to obtain heterologous fusion products. Among the fusion products were some tomato plants that were indistinguishable from the original cultivars with respect to morphology, physiology, and chromosome number (2N = 24) but exhibited various degrees of male sterility (MS): complete lack or malformation of anthers, shrunken pollen, and normal-looking stainable pollen that could not germinate. The MS thus induced in five cultivars of different growth types, including one of subspecies *L. esculentum cerasiforme*, was inherited maternally over several generations and is, therefore, cytoplasmically determined MS (CMS). Analysis of mitochondrial DNA revealed that the mitochondrial genome of the CMS hybrids does not contain all elements of the mitochondrial DNA of either parent but includes sequences of a recombinational nature not present in either parent. The CMS hybrids, therefore, possess a true hybrid mitochondrial genome. The same procedure applied to fusion of tomato with *Solanum lycopersicoides* and *Nicotiana tabacum* cells did not produce CMS phenotypes. The advantages of this method over others for generating MS are as follows: (*i*) only one step is required; (*ii*) the nuclear genotype of the cultivar is unaffected; (*iii*) the prospect that cytoplasmic determination allows generation of 100% CMS progenies. The normal-appearing but nonfunctional pollen of certain CMS types might render them attractive to pollinating bumblebees that thus would facilitate production of hybrid seed.**

Male sterility (MS) can be artificially introduced into flowering plants by chemical treatment, by delivery of nuclear and/or organellar genes (1), or by transformation with a chimeric gene composed of an anther-specific promoter and a ribonuclease gene (2). In the production of hybrid seed, the need for MS plants is increasing to avoid manual emasculation of female parents, as ≈40% of the total labor is expended for this task, and to avert contamination by self-pollination. In conventional breeding, however, the use of genic MS requires many generations of backcrossing and selection from original sources. A method to produce MS in one step within a desired cultivar would, therefore, be extremely useful.

Symmetric hybrids between tomato and potato, produced by protoplast fusion (3–5), showed an intermediate cold tolerance (6). Substitution of *Solanum acaule* genome into *Solanum tuberosum* could result in an appreciable increase of frost resistance (7, 8). Subsequently, experiments were initiated to compare different fusion methods in this system. For this purpose tomato protoplasts were inactivated with iodoacetamide (IOA), which damages mitochondria and seems not to affect nuclear genomes, and the fusion partners of *S. acaule* or *S. tuberosum* were treated with high doses of ionizing irradiation, which inactivates nuclear genomes and also prevents callus formation. Products of the resulting fusion should be heterologous hybrids. Close examination of the regenerated offspring revealed the presence of MS.

The method thereby produces MS in one step instead of the many required by conventional breeding. A description of the method and analysis of the progeny form the substance of this report.

MATERIALS AND METHODS

Plant Materials and Culture Conditions. *S. acaule* Hawkes (2N = 4X = 48) was reproduced by self-pollination or vegetatively propagated from tubers for many years in the Max-Planck-Institute for Breeding Research, formerly at Müncheberg, now at Köln-Vogelsang, Federal Republic of Germany. *S. acaule* seeds were aseptically germinated on a wet paper soaked with a half-strength Knop solution (1× Knop solution = 1000 g of H_2O/1.0 g of $Ca(NO_3)$/0.25 g of $MgSO_4 \cdot 7\ H_2O$/0.25 g of KH_2PO_4/0.25 g of KNO_3/traces of $FeSO_4$) and the seedlings were cultured at 24°C with a regimen of 16 h of light (4500 lux) and 8 h of dark. They were then transferred onto modified Shahin TM-5 medium (9), in which concentration of indole-3-butyric acid was increased to 0.5 mg/liter and cultured for an additional 4 weeks with a regimen of 16 h of light (10,000 lux) at 25°C and 8 h of darkness at 15°C. *S. tuberosum* L. cv. Tunica and its dihaploid line (H29) were from the National Institute of Agrobiological Resources (Tsukuba, Japan). *Solanum lycopersicoides* was kindly donated by C. Rick (Davis, CA).

Seeds of *Lycopersicon esculentum* Mill. cv. Sekai-ichi and Kurihara were purchased from Sakata Seed (Yokohama, Japan), and seeds of *L. esculentum* cv. Delicious, Kurihara, PI 341988, and VF-36 and subspecies *L. esculentum cerasiforme* cv. Red Cherry were kindly provided by D. Ishiuchi (Morioka, Japan). The tomato seeds were aseptically sown on a TM-1 medium (9) and the seedlings were cultured at 24°C under a regimen of 16 h of light (4500 lux) and 8 h of darkness.

Fusion of Protoplasts and Culture of Somatic Hybrids. Protoplasts were prepared by a modified method of Shahin (8). Tomato seedlings, 12 days after sowing, were kept at

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Abbreviations: MS, male sterility; CMS, cytoplasmic MS; IOA, iodoacetamide.

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24°C in darkness for 2 days and the cotyledons were cut for protoplast isolation. The cotyledons were incubated with preenzyme treatment (PET) solution (9) before enzyme treatment at 10°C for 6 h. Fully expanded leaves of *S. acaule* were used for protoplast preparation after having been maintained in darkness at 24°C for 2 days. The leaves were then cut into small pieces with a scalpel and irradiated with 1 kGy of γ- or x-ray in the PET solution. Protoplasts of both plants were prepared by exposing leaf tissues to an enzyme solution containing 0.2% Macerozyme R-10 (Yakult Pharmaceutical, Tokyo) and 2% (wt/vol) cellulase Onozuka R-10 (Yakult Pharmaceutical) at 25°C for 16 h and were suspended in W5 solution (10) containing 5 mM Mes (pH 5.8). The mitochondria of tomato protoplasts were inactivated with 10 or 15 mM IOA in W5 solution at 4°C for 15 min. After IOA treatment, the protoplasts were washed twice with W5 solution.

Tomato protoplasts treated with IOA and *S. acaule* protoplasts irradiated with γ- or x-ray were mixed in a 1:1 ratio at a density of 1×10^6 protoplasts per ml. Fusion was carried out by the modified method of Kao *et al.* (11), in which the concentration of polyethylene glycol (PEG) 1540 (Wako Pure Chemical, Osaka) was decreased to 25% (wt/wt) and the concentration of mannitol in the high-pH/high-Ca solution was changed to 0.2 M. The concentration of $CaCl_2$ in the washing solution was increased to 75 mM. After fusion, the protoplasts were cultured according to Shahin (9).

Examination of Pollen Fertility. Fertility of freshly collected pollen was examined for the stainability of pollen grains with acetocarmine or fluorescein diacetate (19) and, subsequently, for the germination rate of pollen grains on an artificial medium, as described in Brewbaker and Kwack (12), solidified with 0.2% Gelrite (Merck, Kelco Division) within 2 h. Pollen on stigmas was also examined 24 h after pollination by the method of Kho and Baer (13).

Analysis of Mitochondrial DNA (mtDNA). Mitochondria were isolated from leaves of fully grown plants. The leaves were homogenized in extraction buffer [0.44 M sucrose/50 mM Tris·HCl, pH 7.59/3 mM EDTA/0.1% polyvinylpyrrolidone/0.2% bovine serum albumin/1 mM 2-mercaptoethanol] with a blender. The homogenate was filtered through three layers of cheesecloth and then through one layer of Miracloth and then centrifuged at $1400 \times g$.

The supernatant was centrifuged at $2200 \times g$ for 10 min and then at $15,000 \times g$ for 10 min, and the resulting pellet was suspended in a Tris·HCl (pH 7.5) containing 25 mM $MgCl_2$, 0.44 M sucrose, and DNase I (200 μg/ml), and kept on ice for 30 min. Subsequently, $MgCl_2$ and DNase I were added to the suspension to 40 mM and 300 μg/ml, respectively, and the enzyme treatment was continued for another 30 min. The reaction was stopped with the addition of EDTA to 50 mM. Mitochondria were collected by centrifugation after dilution with washing buffer (50 mM Tris·HCl, pH 7.5/20 mM EDTA/0.1% polyvinylpyrrolidone). This washing was repeated four times. The resultant pellet of mitochondria was lysed in 1% sarkosyl/20 mM EDTA/proteinase K (200 μg/ml)/50 mM Tris·HCl, pH 8.0, at 37°C for 1 h. After mtDNA was extracted with phenol and phenol/chloroform/isoamyl alcohol, 24:24:1 (vol/vol), it was precipitated with ethanol and purified by CsCl density gradient centrifugation at $140,000 \times g$ for 16 h. The mtDNA was digested with *Hin*dIII, *Pst* I, *Sal* I, *Bam*HI, and *Eco*RI and analyzed by agarose gel electrophoresis. We thank R. Hiesel and A. Brennicke for these analyses.

RESULTS

***S. acaule* as a Donor of mtDNA.** Neither tomato protoplasts treated with 10 or 15 mM IOA nor *S. acaule* protoplasts irradiated with 1 kGy of γ- or x-ray formed calli. Table 1 shows the result of a fusion experiment of Sekai-ichi tomato protoplasts with *S. acaule* protoplasts, in which the four possible combinations of IOA treatment and irradiation were carried out. From this experiment, 88 calli were obtained from 2×10^6 tomato and *S. acaule* protoplasts on TM-3 medium. Although partial organ formation was observed in 9 calli upon their transfer onto the TM-4 medium, three shoots that ultimately formed roots (MSA, MSB, and MSC; Table 2) were obtained on TM-5 medium and grown to maturity in a greenhouse. There were no significant morphological differences in Sekai-ichi, MSA1, and MSB2 tomatoes. Their chromosome number was 24. Only MSC plants with 48 chromosomes looked like the tetraploid Sekai-ichi tomatoes produced by colchicine treatment (data not shown).

Table 1. Asymmetric somatic fusion of Sekai-ichi protoplasts with *S. acaule* protoplasts

Treatment		Protoplasts used, no.	Calli formed, no.	Calli regenerated, no.
Recipient (Sekai-ichi)	Donor (*S. acaule*)			
IOA (10 mM)	γ-ray (1 kGy)	6.4×10^5	29	2
IOA (10 mM)	X-ray (1 kGy)	4.0×10^5	13	1
IOA (15 mM)	γ-ray (1 kGy)	6.4×10^5	17	4
IOA (15 mM)	X-ray (1 kGy)	4.0×10^5	29	2

When MSA1 was pollinated with Sekai-ichi tomato pollen, the plants bore fruits of the same size as Sekai-ichi tomatoes, whereas the self-pollinated MSA1 yielded only smaller fruits with no seeds. MSA1 pollen was found to be incapable of pollinating Sekai-ichi tomatoes. This pollen sterility was accompanied with very low *in vitro* germination rate of null to 1%, although the staining of MSA1 pollen with acetocarmine and fluorescein diacetate did not show it to be much different from Sekai-ichi tomato pollen (Fig. 1). MSB pollen was stained with acetocarmine in the range of 7 and 44%, whereas MSC pollen was stained at 0% (Table 2).

Pollen from either MSB or MSC did not germinate. Coincidently, pollen from MSA1 and MSB2 did not germinate on stigmas of tomato cultivars Sekai-ichi, Morioka 16, VF-36, or PI 342988 or on stigmas of regenerated MSA, MSB, and MSC plants (Table 3). The number and weight of seeds in fruits of MSA1 progeny pollinated with Sekai-ichi pollen [148 ± 43 per fruit and 2.81 ± 0.66 g per 1000 seeds (mean ± SEM), respectively] did not differ statistically from those of self-pollinated Sekai-ichi (161 ± 63 per fruit and 3.11 ± 0.79 g per 1000 seed, respectively); furthermore, length and thickness of cotyledons of 3-week-old seedlings from these seeds (4.80 ± 0.69 cm and 0.60 ± 0.04 mm, respectively) did not differ statistically from those of self-pollinated Sekai-ichi (5.00 ± 0.49 cm and 0.59 ± 0.03 mm, respectively).

Inheritance of MS. Pollen fertility of the offspring of MSA1, MSB1, and MSB3 pollinated with Sekai-ichi tomato pollen was examined and compared with that of self-pollinated

Table 2. Pollen viability of plants from MSA, MSB, and MSC

Plant	Staining rate, %	Germination rate, %
Sekai-ichi	94.9 ± 4.6	48.9 ± 23.4
MSA1	93.6 ± 5.8	0
MSA2	90.5 ± 8.5	0.8 ± 0.9
MSB1	8.5 ± 5.9	0
MSB3	47.1 ± 25.2	0
MSB4*	0	0
MSC4	0	0
MSC5	0	0
MSC14	0	0
MSC23	0	0

Viability of pollen from regenerated plants was examined by the staining with acetocarmine and by germination on the artificial medium. Data are mean±SEM.
*This plant was stamenless.

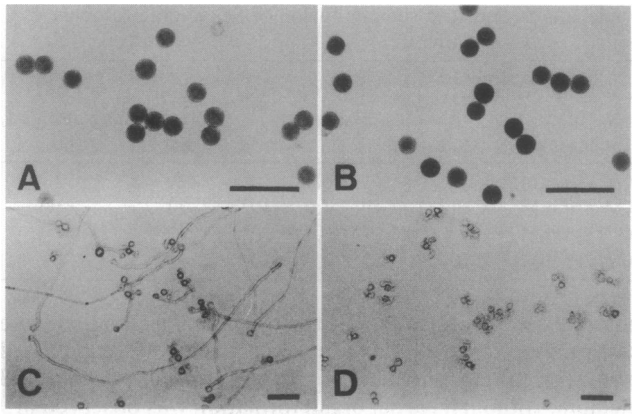

FIG. 1. Staining of pollen with acetocarmine and germination of pollen. Staining of pollen from Sekai-ichi tomatoes (*A*) and male-sterile MSA1 tomatoes (*B*) with acetocarmine. The pollens from MSA1 and Sekai-ichi were stained at the same frequency. Germination of pollen was examined by the method of Kho and Baer (13). The germination rate of Sekai-ichi (*C*) and MSA1 (*D*) pollen was 20 and 0%, respectively. (Bars = 100 μm.)

MSA1 (Table 4). Although the pollen from MSA1 and MSB3 progeny was stained at >90% and pollen of MSB1 was stained at ≈6%, neither of these pollen germinated on the test medium. This experiment demonstrated that the attained MS was maternally transmitted at least up to four generations by pollination with Sekai-ichi tomato pollen without any segregation. MSA2, which was a partially male-fertile plant, produced seeds by selfing; its progeny were partially male-fertile and their pollen germinated on the test medium at the same rate as MSA2 pollen.

Other Tomato Cultivars. Protoplasts from other tomato cultivars of Delicious, VF-36, Kurihara, and the subspecies *L. esculentum cerasiforme* cv. Red Cherry were treated with IOA and fused with irradiated *S. acaule* protoplasts. Somatic hybrid plants were regenerated from the fusion products and displayed morphological characteristics that resembled either tomato plants or hybrid plants. When the tomato-like plants were grown to maturity in a greenhouse, they showed MS and had a chromosome number very similar to that of the Sekai-ichi hybrids described above (data not shown). Some of these progeny (PKE1 from Kurihara, PDA1 from Delicious, and PRB3 from Red Cherry) that are male-sterile and some (PKB2 from Kurihara, PDE2 from Delicious, and PRA1 from Red Cherry) that are partially male-fertile may allow the comparison of different percentages of MS with mtDNA structure.

Table 3. Germination of pollen on stigma observed under a fluorescence microscope

Stigma	Stamen	Pollen germination
Sekai-ichi*	Sekai-ichi	+
MSA1	Sekai-ichi	+
MSA2	Sekai-ichi	+
MSB1	Sekai-ichi	+
MSB3	Sekai-ichi	+
MSB4	Sekai-ichi	+
MSC4	Sekai-ichi	+
Sekai-ichi*	MSA1	−
Morioka 16*	MSA1	−
VF-36*	MSA1	−
PI 341988*	MSA1	−
MSA1	MSA1	−
MSB3	MSA1	−
MSC5	MSA1	−
Sekai-ichi*	MSB3	−
Morioka 16*	MSB3	−
VF-36*	MSB3	−
PI 341988*	MSB3	−
MSB3	MSB3	−

+, Pollen germinated; −, pollen did not germinate.
*These tomato cultivars are male- and female-fertile.

Analysis of mtDNA. mtDNA of MSA1 was examined by restriction fragment polymorphism analysis to determine its origin (carried out by R. Hiesel and A. Brennicke). When mtDNA was cut with several restriction enzymes (*Bam*HI, *Hin*dIII, *Pst* I, and *Sal* I) and electrophoresed on agarose gels, fragments showed variable similarity to fragments of the fusion partners (Figs. 2 and 3). The restriction enzyme digestion patterns in Figs. 2 and 3 revealed that mtDNA of the hybrid (L+S) was derived either from tomato mtDNA or from *S. acaule* mtDNA, since common mtDNA fragments between the hybrid and tomato or those between the hybrid and *S. acaule* were observed. However, this does not necessarily mean that mtDNA of the hybrid was the simple amalgamation between mtDNAs of tomato and *S. acaule*, since there were some fragments that were observed in parental mtDNA of tomato or *S. acaule* but were not observed in the mtDNA of the hybrid. It should be noted that there were bands that were observed solely in the mtDNA of the hybrid (Fig. 2, band 5 in the *Hin*dIII digest) but not in the mtDNA of both fusion partners. Such bands were observed also in the *Bam*HI and *Sal* I digests (Fig. 3). Such fragments could be derived from the unique recombination events between mtDNAs of both parents that formed the hybrid mtDNA.

Analysis of mtDNA from male-sterile and partially male-fertile plants cut with *Eco*RI showed that the MS plants (MSA1, PKE1, and PRB3) had more fragments from *S. acaule* than did the male-fertile plants (MSA2, PKB2, and PRA1) (data not shown).

***S. tuberosum* as a Donor of mtDNA.** IOA-treated protoplasts from Sekai-ichi tomato were fused with irradiation-inactivated protoplasts from the dihaploid H29 potato. From 128 calli obtained from fusion products, three male-sterile tomato lines (116/01, 116/07, and 116/12) were selected from a single callus. Although 40–70% of pollen of these plants was stained with acetocarmine, none of the pollen germinated in the test medium. These plants did not set fruits by selfing, but bore fruits and seeds by pollination with Sekai-ichi tomato pollen. They had 24 chromosomes and showed no observable morphological difference from Sekai-ichi tomato. Thus it was concluded that potato could be used as a donor of cytoplasmic MS (CMS) in tomato. Similar results were obtained from fusions with the tetraploid Tunica potato. This MS was inherited maternally, and the regenerated male-sterile plants retained fragments of mtDNA of fusion partners and fragments not observed in the fusion partners that could be recombination products (data not shown). These results suggest that MS could be caused by the recombination of tomato mtDNA and potato mtDNA.

***S. lycopersicoides* and *Nicotiana tabacum* as Donors of mtDNA.** From the fusion products between Sekai-ichi tomato and *S. lycopersicoides*, 29 regenerated plants were morphologically indistinguishable from Sekai-ichi tomato and all were male-fertile. Examination of mtDNA of three of these plants revealed that two plants possessed mtDNA from both parents, and one plant had only Sekai-ichi tomato mtDNA (data not shown). Forty-one lines derived from 10 calli of the fusion products of Sekai-ichi tomato and *N. tabacum* protoplasts were all pollen-fertile. Correspondingly, the restriction enzyme patterns of mtDNA from 4 lines were the same as the pattern from tomato mtDNA (data not shown).

Table 4. Examination of the pollen from the progeny of MSA and MSB

Plant	Staining rate, %	Germination rate, %
Sekai-ichi (Si)	94.9 ± 4.6	48.9 ± 23.4
MSA1 × Si	95.2 ± 3.1	0
MSA1 × Si × Si	94.5 ± 5.1	0
MSA1 × Si × Si × Si	94.8 ± 4.4	0
MSA1 × Si × Si × Si × Si	95.3 ± 4.5	0
MSA2 × MSA2	90.8 ± 6.9	1.6 ± 1.8
MSA2 × Si	93.1 ± 5.7	1.9 ± 2.3
MSB1 × Si	5.5 ± 2.7	0
MSB3 × Si	95.6 ± 1.5	0

Male-sterile plants were pollinated with Sekai-ichi (Si) pollen. Partially male-fertile MSA2 was self-pollinated or pollinated with Sekai-ichi. The stainability of pollen with acetocarmine and the germinability of pollen on the germination medium were examined. In each generation at least 20 plants were examined from which more than three flowers were collected.

DISCUSSION

In spite of the identification of ≈50 nuclear genes that determine MS, the quest for CMS in this crop has heretofore been unsuccessful, to our knowledge. As described above, our method of introducing altered mtDNA succeeds in generating not only CMS but also a surprising variety of CMS phenotypes from absence or great distortion of anthers to normal anthers with nonfunctional pollen. This result could be due to the recombination of tomato mtDNA with mtDNA of the donor plants. Some specific recombination event seems to be responsible for this MS, a detailed study of which has not yet been accomplished. Since this genetic trait was stably inherited by pollination with pollen of normal tomatoes, this method of MS production can be widely used for the production of hybrids in plant breeding. Thus far hybrid seeds have generally been produced by laborious and expensive manual emasculation of maternal plants. Alternatively, when the MS was induced by the introduction of genes from other plants by conventional means, backcrossing and selection were required to eliminate undesirable genes. In contrast, our method of MS production did not change the composition of nuclear genomes of the cultivated tomato and thus the male-sterile plants could be used for practical breeding objectives without these delays. Although the CMS system would be supplemented by restorer genes in the hybrid cultivars and their incorporation into the male parents, we have detected weakly effective restorer genes in cultivars of VF-36 and Red Cherry in a preliminary experiment. When MSA1 was pollinated with Sekai-ichi, Kurihara, VF 36, or Red Cherry pollen, only progeny from MSA1 × VF-36 and MSA1 × Red Cherry set several seeds by open pollination. Furthermore, when 46 progenies of MSA1 × Red Cherry × Red Cherry were cultivated in an open field to examine the ability of seed set, 35 plants out of the 46 progenies bore fruits, containing a mean of 6.2 seeds, whereas self-pollinated Sekai-ichi and Red Cherry bore fruits, containing 154 and 47 seeds, respectively. In the summer of 1991, G.M. in cooperation with H. Uhrig (Max-Planck-Institut für Züchtungsforschung, Cologne–Vogelsang, F.R.G.) found 7 fruits with different numbers of seeds in ≈2000 fruits from 150 plants cultivated in an insect-proof greenhouse. It has to be determined by conventional genetical methods whether these seeds originated by restorer genes developed by spontaneous

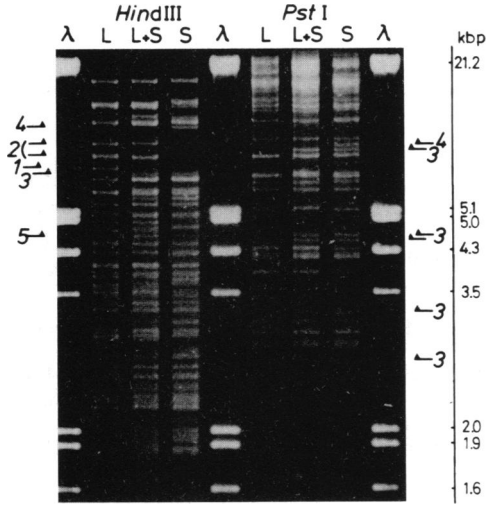

FIG. 2. Restriction endonuclease digestion patterns of mtDNAs of *L. esculentum* cv. Sekai-ichi, *S. acaule*, and male-sterile MSA1 with *Hin*dIII and *Pst* I. mtDNAs of *L. esculentum* (lanes L), MSA1 (lanes L+S), and *S. acaule* (lanes S) were digested with *Hin*dIII or with *Pst* I as indicated. Digestion fragments were classified to five groups that are noted to the left and right. Groups: 1, bands observed only in mtDNA of *L. esculentum*; 2, bands observed in mtDNAs of *L. esculentum* and the hybrid; 3, bands observed in mtDNAs of the hybrid and *S. acaule*; 4, bands observed only in mtDNA of *S. acaule*; 5, bands observed solely in the mtDNA of the hybrid. Thus, although mtDNA of the hybrid was composed of mtDNAs of both fusion partners, the mtDNA was not a simple amalgamation of both partners. A band observed only in the hybrid could have been produced by recombination between mtDNAs of *L. esculentum* and *S. acaule*. λ phage DNA double-digested with *Eco*RI and *Hin*dIII was used as molecular markers (lanes λ). We thank R. Hiesel and A. Brennicke for these analyses.

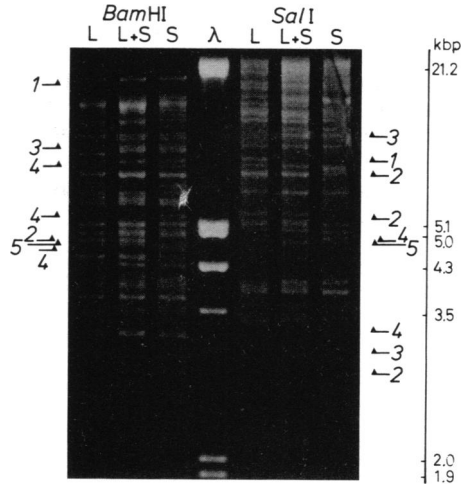

FIG. 3. *Bam*HI and *Sal* I restriction endonuclease digestion patterns of mtDNAs of *L. esculentum* cv. Sekai-ichi, *S. acaule*, and male-sterile MSA1. mtDNAs of *L. esculentum* (lanes L), MSA1 (lanes L+S), and *S. acaule* (lanes S) were digested with *Bam*HI or with *Sal* I as indicated. Bands are as described in Fig. 2. kbp, Kilobase pairs. We thank R. Hiesel and A. Brennicke for these analyses.

mutation or by the variability of CMS. Although numerous nuclear genes for MS and combinations with cytoplasmic factors have been described (1), these methods have not been used very frequently because of the accompanying undesirable genes from the highly bred cultivars.

Many of the problems of utilizing MS in tomato hybrid seed production can now be overcome, since the nuclear genome composition of the five tomato cultivars we describe was not changed. Furthermore, our method might allow the use of bumblebees to assist in hybrid seed production, as our sterile pollen could not be morphologically distinguished from fertile pollen. Then the production of hybrid seed and the search for restorer mutants would be facilitated. Although one-step production of CMS tomato has been established by the fusion of tomato protoplasts with protoplasts of *S. acaule* or potato as an mtDNA donor, the next question is can our asymmetric fusion be applied to other plant species in which the introduction of MS is highly desirable. In this context understanding of the mechanism of the production of this type of male-sterile plants would be helpful. The following two examples suggest the answer. Fusion of IOA-treated tomato protoplasts with irradiated protoplasts from *S. lycopersicoides* or *N. tabacum* did not produce male-sterile tomatoes. The fusion products of tomato with *S. lycopersicoides* retained hybrid mtDNA but did not show MS. Thus *S. lycopersicoides* mtDNA does not function like *S. acaule* or potato mtDNA and *S. lycopersicoides* could thus be more phylogenetically related to *Lycopersicon* than the nomenclature suggests, as Rick (14) noted from other evidence. Bonneman *et al.* (15) reported that tomato hybrids with *Lycopersicon pennellii* were fertile. This result could be interpreted as above for the *S. lycopersicoides* hybrids; therefore, the mtDNA donor should be at some phylogenetical distance from the recipient. If this assumption is correct, the reason for the production of male-fertile tomato by asymmetric fusion between tomato and *N. tabacum* should be different than between tomato and *S. lycopersicoides*, as the fusion products did not contain hybrid mtDNA. The presence of mtDNA from tobacco should have existed at least during the initial stage of culture after fusion and subsequently must have been lost by some mechanism that has not yet been identified. The stages in which tobacco mtDNA was lost could be at the callus stage or in the meristem of differentiating plants, as has been demonstrated by Sacristán and Melchers (16) who observed that tobacco mosaic virus was eliminated from the meristem of tobacco cultivated for a long time in a callus culture. These two results suggest that fusion partners for the production of MS should not be too closely or too remotely related.

However, it remains to be resolved whether the band observed after restriction endonuclease digestion of mtDNA that seems to be responsible for MS in tomato would have any relationship with the *S-pcf* (petunia CMS-associated fused) gene of *Petunia hybrida* (17) or with the *Urf13-T* gene of *Zea mays* (18), both of which have been proposed to be responsible for CMS in the respective species.

Note Added in Proof. In the mean time we produced for practical plant breeding (*i*) the MS of cultivar MN and (*ii*) of cv. Ohmiya, both Tokita Co., Japan, and (*iii*) MS of cv. 0670038, Peto Seed, California, USA.

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