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Somatic Hybrids between *Lycopersicon esculentum* and *Lycopersicon chmielewskii*

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Abstract

Interspecific somatic hybrids were obtained by electric protoplast fusion of cotyledon protoplasts of *Lycopersicon esculentum* Mill. cv. Kyoryokutoko treated with iodoacetamide (IOA) and suspension-culture-derived protoplasts of *L. chmielewskii* (LA1330). A multiple-step selection procedure was used in selecting the hybrids depending on IOA treatments and a culture medium that only allows cotyledon protoplasts to regenerate. Plants were regenerated on the calli derived from fused protoplasts. After acclimation, the hybrid plants with fruits through self-pollination were obtained. Identification of the regenerated putative hybrid plants was carried out by morphological, chromosomal and random amplified polymorphic DNA (RAPD) analysis, as well as restriction fragment length polymorphism (RFLP) comparison of parental and hybrid plants. The hybrids showed unique morphologies in leaf, flower and stem compared with that of two fusion partners, 2n = 4x = 48, 47, 69-72 chromosomes, both of the parental-specific RAPD bands, and genomic recombination and elimination occurred in mitochondria level. Cell fusion technique shows its new potential in foreign gene transformation between *L. esculentum* and *L. chmielewskii*.

Key words: Iodoacetamide (IOA), *Lycopersicon chmielewskii*, *Lycopersicon esculentum*, Random amplified polymorphic DNA (RAPD), Restriction fragment length polymorphism (RFLP), Somatic hybrids.

Introduction

Lycopersicon esculentum, tomato is well known as a model plant for cytological and molecular research. It is the number one of production in volume among vegetable crops in the world (FAO internet database, 1998). In *Lycopersicon* genus, there are nine species, and they are grouped as two, *esculentum*-complex (seven species) and peruvianum-complex (two species) (Rick and Yoder, 1988). The *peruvianum*-complex (*L. peruvianum* and *L. chilense*) is a source of agronomic traits, such as resistance to various diseases and pests. However, the *peruvianum*-complex is also distinctly related to the tomato. Up to now, the interspecific hybrids between the two groups have been obtained by embryo rescue (Smith, 1944; Chen and Imanishi, 1991; Chen and Adachi, 1992, 1996). Somatic hybrids have also been obtained in *L. esculentum* and *L. peruvianum* (Wijbrandi *et al.*, 1990; Chen and Adachi, 1998; Chen *et al.*, 1998), and in *L. esculentum* and *L. chilense* (Chen and Adachi, 1998; Chen *et al.*, 1998). In the same *esculentum*-complex group, as unilateral-incompatibility exits, the interspecific hybrids were also difficult to obtain. Somatic hybrids have been done in the same group, such as *L. esculentum* and *L. pennellii* (O'Connell and Hanson, 1985). The purpose of the above experiments was mainly to transfer the resistance to various diseases and pests from wild species to tomato.

Recently, cell fusion is reused again in some usages. For example, transformation of apomixis genes into rice plants was carried out by cell fusion (Yan and Zhang, 2001). In this point, cell fusion techniques can be used not only in breeding between remote accessions but also in partial gene transfer. As chemical treatment of iodoacetamide (IOA) (Ozias-Akins *et al.*, 1986; Terada *et al.*,

1987) or gamma-irradiation (Ralushnyak *et al.*, 1991) can inactive one partner, the possibility becomes practical and actual that some special components could be transferred from one cell into another.

In *Lycopersicon*, symmetric and asymmetric somatic hybrids have been made (Lefrancois *et al.*, 1993; Wolters *et al.*, 1994; Chen *et al.*, 1998). Somatic embryogenesis from protoplasts is an attractive possibility for the propagation of somatic hybrids. In addition, transgenic plants or plant recovery of chemical- or radiation-induced mutations are more readily possible using this pathway because somatic embryos predominantly arise from single cells (Haccius, 1978).

While sexual hybrids of most species carry only the maternal organelle genomes, somatic hybridization can result in transmission of either parental organelle genome or novel organelle genomes to the regenerated plants (O'Connel and Hanson, 1985). *L. chmielewskii*, a small yellow-green fruited wild relative of the cultivated tomato, carries high soluble solids and sucrose accumulation in fruits, and contains approximately twice the soluble sugar concentration of *L. esculentum* fruit. This species belongs to *esculentum*-complex group and is sexually compatible with *L. esculentum* (Rick *et al.*, 1976). Up to now, genes for high solids have been incorporated into tomato via extensive backcrossing and selection (Rick, 1974) and they have been mapped to the middle and terminal regions of chromosome 7 and to terminal region of chromosome 10 (Tanksley and Hewitt, 1988). Further, the effect of introgressed segments of chromosome 7 and 10 from *L. chmielewskii* on tomato soluble solid, pH, and yield has been characterized (Azanza *et al.*, 1994). The major gene, designated *sucr*, for sucrose accumulation was mapped in F_2, F_3, and BC_1F_2 population using a set of 95 informative RFLP and isozyme markers covering the tomato genome (Chetelat *et al.*, 1993). That the *sucr*, a sucrose accumulator gene, controls fruit sugar composition has been identified using linkage analysis, RFLP and PCR detection (Chetelat *et al.*, 1995).

As for introgression of sucrose accumulation and high soluble solids through sexual hybridization of *L. esculentum* x *L. chmielewskii* usually takes long time to finish extensive backcrossing (Rick, 1974; Chetelat *et al.*, 1993), and the crossing is limited by the seasons of growth, using protoplast fusion techniques to regenerate somatic hybrids between *L. chmielewskii* and *L. esculentum* will not only ignore these problems but also provide unique experimental materials and novel organelle genomes for introgression and analysis of *sucr* and high solid genes. In this study, we describe plant regeneration from protoplast fusion between *L. esculentum* and *L. chmielewskii*, and determine the putative somatic hybrids by observation of their morphology, chromosome number, and analysis of restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers.

Materials and Methods

Plant materials and protoplast isolation

Lycopersicon esculentum Mill. cv. Kyoryokutoko (KT), a Japanese variety, and *L. chmielewskii* LA1330 (LC) (kindly provided by Dr. Rick, California U., USA) were used as materials for protoplast fusion. Cotyledons of KT were collected from seedlings of 12 days old and from them the protoplasts were isolated according to previous report (Chen and Adachi, 1994). For LC, leaves of 5-week-old plants were used for callus formation according to previous report (Chen and Adachi, 1998). After the calli were subcultured twice on the same medium at 4-week intervals, fresh friable calli were finely separated and transferred into Uchimiya and Murashige (1974) medium. Suspension culture was established by subculture at weekly intervals. The protoplasts of LC were isolated from the suspension culture as described by Chen *et al.* (1998).

Protoplast fusion

Before fusion, cotyledon protoplasts were treated with 10 mM iodoacetamide (IOA), for 15 min to partially inactivate the mitochondria. Both protoplasts of KT and LC were suspended in TM-2 (Shahin, 1985), respectively, adjusted into 1 x 10^5 ml^{-1}, and mixed with 1:1 proportion. The electric apparatus and the fusion procedure were carried out as described by Sihachakr *et al.* (1989). SSH-2 fusion apparatus (Shimazu, Japan) with a movable chamber (1.6 ml capacity) consisting of parallel electrodes (4 mm apart) was connected to both a function generator and a generator of D. C. square pulses. The mixtures of the protoplasts were placed into the chamber for 5 min to let them go down to the bottom. In order to align the protoplasts, the conditions of 1MHz, 40 V cm^{-1}, 20 s were done. Subsequently, one D. C. square developing 1 kV cm^{-1} for 40 μs was applied to achieve protoplast fusion. After that, protoplasts were placed for 20 min and then moved into centrifuge tubes for spin down. When the protoplasts went automatically down after 20 min rest, the 9 M solution (Zapata and Sink, 1981) was removed and 0.4% gellan gum solution was added to adjust the protoplast density into 1 x 10^5 ml^{-1}. The protoplasts with wrap gellan

gum (Wako, Japan) were drop into TM-2 medium (Shahin, 1985), and cultured at 25 ℃, 3.3 μmol m^{-2} s^{-1} on 16-h photoperiod.

Protoplast culture and plant regeneration

Protoplasts from the two partners were also cultured separately as the control as described before (Chen and Adachi, 1994). After four days plating, the cultures were transferred from 35-mm dishes into 60-mm (Falcon 1007) dishes, and 2 ml of the modified culture medium TM-2S was added. The cultures were kept at 25 ℃, 3.3 μmol m^{-2} s^{-1} on 16-h photoperiod. After eight days the culture was moved into 90-mm (Iwaki, 90 x 20 mm) dishes and added 12 ml the medium. Two weeks later, 5-ml aliquots of the cultures were transferred onto TM-3 agar medium in 90-mm dishes for 2-3 weeks culture at 25 ℃, 33.3 μmol m^{-2} s^{-1} on 16-h photoperiod. The microcalli formed on TM-3 medium were transferred onto TM-4 medium supplemented with 1.0 mg l^{-1} zeatin reboside (ZR) and 1.0 mg l^{-1} indoleacetic acid (IAA). After one month, the developed green calli were selected and moved onto modified TM-4 medium supplemented with 2.0 mg l^{-1} ZR. The somatic embryos were formed on the calli and then they were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 1.0 mg l^{-1} zeatin and 0.1 mg l^{-1} IAA to induce shoots. The shoots were removed from the cultures and rooted on hormone-free MS medium. The rooted plants were acclimated as described by Chen and Adachi (1998) and grown until maturity.

Morphological analysis

Morphological characters of leaf shape, stem branching patterns, rooting abilities, and whole plant styles of the regenerants from the fusion experiments were compared to those of the fusion partners.

Chromosome number

The chromosome number of the fusion partners and 10 of the putative somatic hybrids chosen randomly were counted, and 5 cells per plant were investigated using the root tip observation as described by Chen and Adachi (1998). As the plant regeneration of LC was not obtained, the plants of LC derived from seeds were used for chromosome counts.

RAPD analysis

About 100 μg total DNA was isolated from one g leave by the CTAB method of Honda and Hirai (1990) from KT, LC and the putative somatic hybrids. Twenty ng DNA gave an observable band pattern, and this amount was used as a DNA template for PCR. The ten-mer kit Operon V series, and *Tth* DNA polymerase (TOYOBO, Japan) were used for PCR. The PCR mixture consisted of 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 80 mM KCl, 1.25 mM of each dNTP (Pharmacia), 500 μg ml^{-1} bovine serum albumin, 0.1% EDTA, and 0.1% Triton-X. It was treated at 94 ℃ for 4 s, 34 ℃ for 30 s, and 72 ℃ for 60 s over 44 cycles. Amplification products were separated by electrophoresis at 50 V for 30 min on 1.0% agarose gel, stained for 15 min in 10 mg ml^{-1} ethidium bromide solution and photographed under UV light.

RFLP analysis

Total DNA was isolated from leaves of KT, LC and the putative somatic hybrids as described by Honda and Hirai (1990). The DNAs were digested with the restriction enzymes *Bam*HI, *Xba*I and *Dra*I at 37 ℃, overnight. The probes of *pRR*217 (rice nuclear rRNA gene), *cytb* (wheat mitochondrial genes), *atp*A and *cox*II (pea mitochondrial genes) (kindly provided by Dr. Hirai of The University of Tokyo) were used in Southern hybridization (Southern, 1975).

Results and Discussion

In totally, 50-60% of the fusion frequency was obtained after electric fusion. For two different kinds of protoplasts, green in color (KT) and white (LC) were used for fusion (**Fig. 1A, B**), we were able to visually identify heterokaryons (**Fig. 1C**). About 25% of the fusion products were heterokaryons. The collapse of cotyledon protoplasts after IOA treatment could be used as preselection. The use of cotyledons as a source of protoplasts (Chen and Adachi, 1994, 1998) has also the other advantages: short culture time (10-12 days), uniformity of donor source for protoplast isolation under controlled conditions, high yields of protoplasts per unit of tissue when compared to leaves, pigmentation of the protoplasts as useful marker for preselection of somatic hybrids at an early stage in protoplast fusion process.

Four to five days later, the fused protoplasts were divided two times (**Fig. 1D**). On the other hand, KT protoplasts did not divide because of IOA treatment, and LC protoplasts divided followed by colony formation. In the mixture of KT and LC protoplasts, only LC protoplasts divided. The establishment of an efficient preselection system for putative somatic hybrids before cell division or colony formation, is important to avoid screening large number of calli or plants derived from fusion experiments. Some

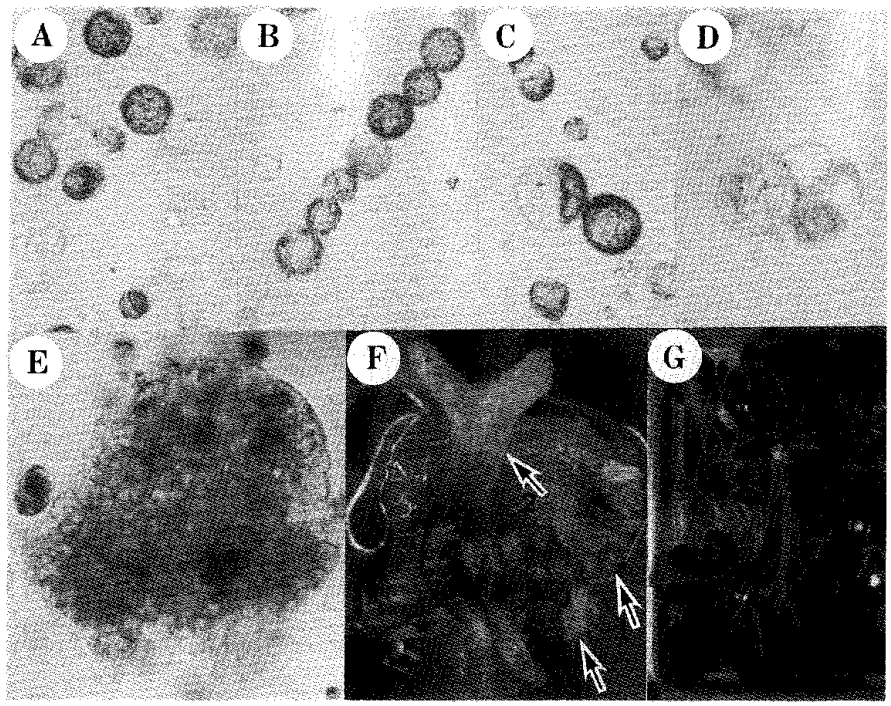

Fig. 1 Somatic embryogenesis and plant regenernation from protoplast fusion between *L. esculentum* and *L. chmielewskii*. (A) A mixture of protoplasts isolated from cotyledon and suspension culture before fusion. (B) Formation of a pearl chain of protoplasts. (C) Fusing protoplasts. (D) Cell division of fused protoplast containing chlorophyll. (E) Callus formation. (F) Somatic embryogenesis on greenish calli with globular- (arrow 1), torpedo-stage (arrow 2) embryos and single cotyledon (arrow 3). (G) Plant regeneration from somatic embryos.

attempts using IOA treatments have been carried out in protoplast fusions with *Nicotiana* (Medgyesy *et al.*, 1980; Sidorov *et al.*, 1981), carrot (Cella *et al.*, 1983) and *Pennisetum americanum* (L.) K. Schum (+) *Panicum maximum* Jacq. (Ozias-Akins *et al.*, 1986). In addition, the optimal IOA treatment depends upon the donor materials used, for instance, 15 min, 10 mM in the dark at 4 ℃ was found to be optimal to prevent any cell division of cotyledon protoplasts of *L. esculentum* KT (Chen and Adachi, 1998). So in this study the same condition of IOA treatment was used here.

After two weeks of plating on TM-3, colonies were formed (**Fig. 1E**). When their sizes reached over 2 mm in diameter, they were transferred onto TM-4 for green callus formation. Somatic embryogenesis with globular-, torpedo-stage embryos and single cotyledons was observed on modified TM-4 medium (**Fig. 1F**). In total, 18 of the complete plants were obtained on hormone-free MS medium after the somatic embryos were transferred (**Fig. 1G**). All the plants were acclimated successfully. They were flowering (**Fig. 2B**), and in two plants, in total three fruits were bore in self-pollination. When the fruits aged about 5 weeks with green color were dissected, only some empty and immature seeds were found. When the pollens were used to germinate, all of the pollens germinated (data not show). The fact that the matured seeds were not obtained may mean that post-fertilization barriers, e.g. embryo abortion, or endosperm collapse, were involved in seed development (Chen and Adachi, 1992, 1996), even the self-pollination and/or fertilization was successful.

Morphological analysis

Morphological analysises of somatic hybrids have been carried out in *Lycopersicon* genus (Kinsara *et al.*, 1986; Sakata *et al.*, 1991; Chen and Adachi 1998; Chen *et al.*, 1998). However, in *L. esculentum* and *L. chmieleskii*, there were no reports on identification of somatic hybrids. In this study, the putative somatic hybrids grew more vigorous than their fusion partners (**Fig. 2A, B, C**), both in vitro and in vivo. Significant differences in leaf morphology were observed between somatic hybrids and their fusion parents (**Fig. 2D**). The leaves of the hybrids showed few wrinkles, no leaflets, and shorter hairs compared to the KT (**Table 1**). The stem thickness was almost between their fusion partners (**Table 1**). The flowers of the hybrids were similar to *L. chmielewskii* with thin yellow color and 5

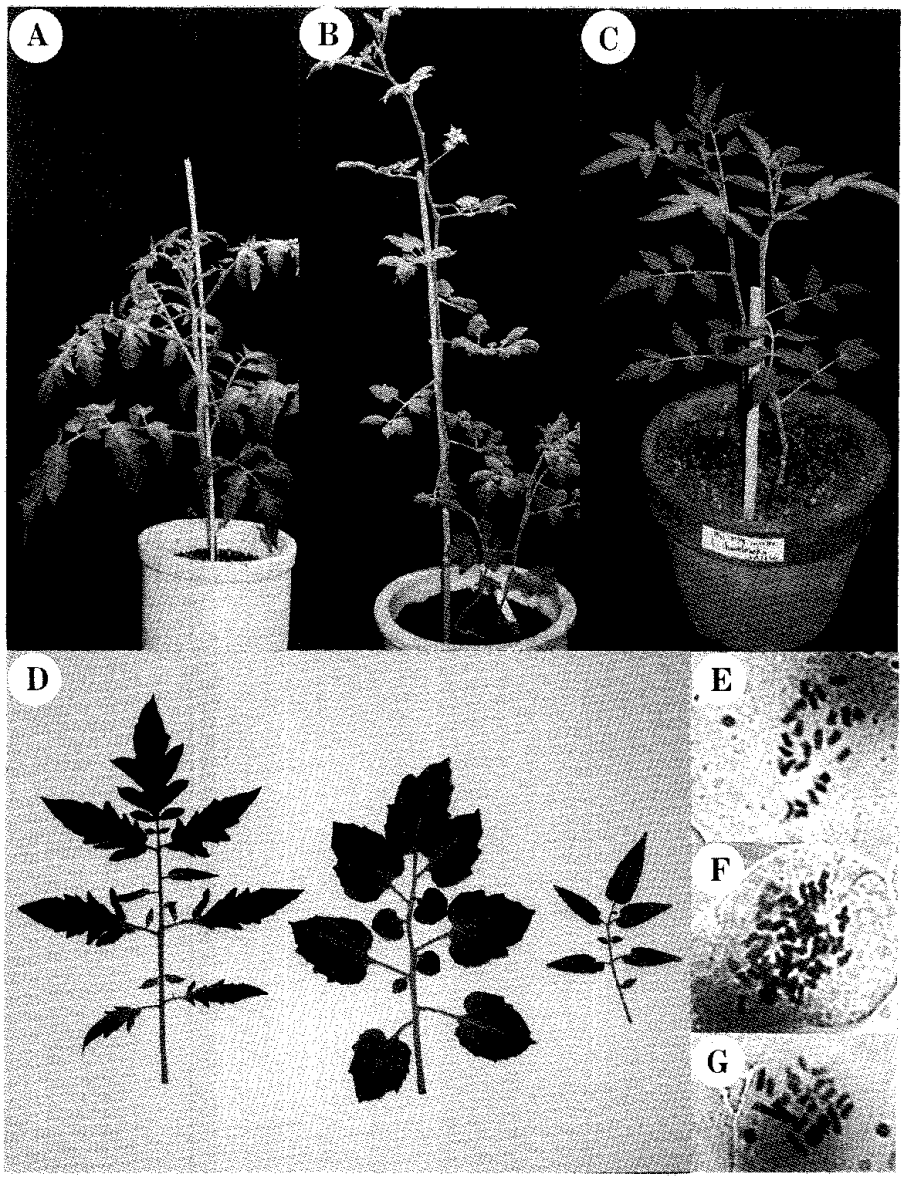

Fig. 2 Morphological and chromosome observations of *L. esculentum*, *L. chmielewskii* and the putative somatic hybrids. (A) Plant of *L. esculentum* cv. Kyoryokutoko (E). (B) The putative somatic hybrid of E (+) C. (C) Plant of *L. chmielewskii* LA1330 (C). (D) Leaves of E (left), E (+) C (middle) and C (right). (E, F, G) Chromosome numbers of E (2n = 2x = 24), E (+) C (2n = 4x = 48) and C (2n = 2x = 24), respectively.

Table 1. Morphological observation from somatic hybrids (five plants) and their fusion partners (five plants per strain invesigated)

	Stem	Hairs	Flower	Leaf
Lycopersicon esculentum (Kyoryokutoko)	Upright, deep green, Φ 20-50 mm	Some, length 3-5 mm	Yellow, 6 pieces of petals, lengwith 30mm	Many deep wrinkles, with leaflets, long and narrow shape
Lycopersicon chmielewskii (LA1330)	Upright, thin green, Φ 15-16 mm	None	Thin yellow,5 pieces of petals,length 20mm	Few shallow wrinkles, no leaflet, long and narrow shape
L. esculentum(+)*L. chmielewskii*	Upright, thin green, Φ 20-28 mm	Some, length 1-1.5 mm	Thin yellow,5 pieces of petals,length 28mm	Few shallow wrinkles, no leaflet, wide shape

pieces of petal, but their length (28 mm) more similar to *L. esculentum* (30 mm) as indicated in **Table 1**.

Chromosome number

The chromosome number of KT and LC was 2n = 2x = 24 (**Fig. 2E** and **G**). For the chosen putative somatic hybrid plants of KT (+) LC, 6 of 10 plants carried 48 chromosomes (**Fig. 2F**). For the other plants, 1 plant had 47 chromosomes, and 3 plants carried between 69-72 chromosomes. No putative somatic hybrids had chromosome counts of 2n = 2x = 24. The putative hybrids with 47-48 chromosomes approximated the expected tetraploid (or aneuploid) level (2n = 4x = 48 chromosomes). That with 69-72 chromosomes approximated the hexaploid (or aneuploid) level (2n = 6x = 72). However, differences in chromosome morphology among the chromosomes in these plants could not observed using this method.

Chromosome elimination was reported in somatic hybridization with radiation (Wijbrandi *et al.*, 1990) and protoplasts were suspension cell derived (O'Connell and Hanson, 1985). Ozias-Akins *et al.* (1986) reported that how IOA treatment affects the chromosome during protoplast fusion in not yet clear. We have obtained a large proportion of aneuploid and hexaploid regenerants when using both suspension-cell-derived protoplasts and IOA-treated protoplasts during cell fusion. In general, IOA was used as inhibitor to inactive the cytoplasm of one partner in asymmetric protoplast fusion. It is suggested that the appearance of aneuploid and hexaploid plants may be related to the protoplasts-derived from suspension culture of LC, as cotyledon protoplasts of KT as mesophyll (leaf, cotyledon) protoplasts, in general, contribute to relatively stable chromosome number in the majority of the putative somatic hybrids.

RAPD analysis

Of the 20 primers of Operon V series used, only OPV-03 gave clear partner-specific polymorphisms. **Fig. 3** showed some examples. The specific bands of *L. esculentum* (ca 2,000bp) and *L. chmielewskii* (ca 1,000bp) were identified, and the putative hybrids contained the bands of both partners (**Fig. 3**).

RAPD markers have been used to screen somatic hybrids in some crops, for example, *Solanum tuberosum* and *S. brevidense* (Xu *et al.*, 1993), *L. esculentum* and *Solanum lycopersicoides* (Hossain *et al.*, 1994), and *L. esculentum* and *L. peruvianum* (Chen *et al.*, 1998). The fact that different band patterns were observed when using the same one primer indicates a wide range of genetic changes among the hybrids. That may be due to somaclonal variation (Xu *et al.*, 1993), and IOA treatment (Ozias-Akins *et al.*, 1986). Other possible explanations for these changes are variation in chromosome number (Karp, 1991), and re-arrangement and amplification of organelle DNA (Rode *et al.*, 1988). However, using different primers, it was understood that all of the putative somatic hybrids tested carried both parental-specific bands.

RFLP analysis

Four kinds of probes were used in this study.

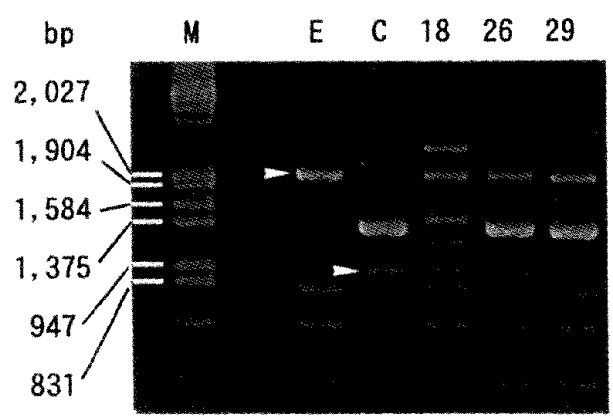

Fig. 3 RAPD analysis of *L. esculentum*, *L. chmielewskii* and the putative somatic hybrids. Lane M, marker; Lane E, *L. esculentum* cv. Kyoryokutoko; Lane C, *L. chmielewskii* LA1330; Lanes 18, 26 and 29, the putative somatic hybrids of E (+) C. Prominent bands specific to each species are marked by arrows. The Operon-V03 primer was used.

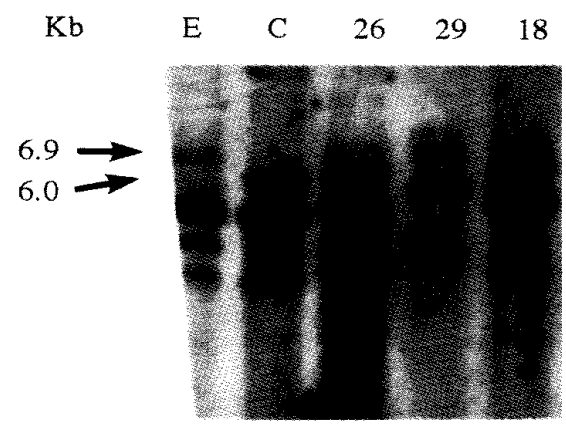

Fig. 4 RFLP of the putative somatic hybrids (Lanes 26, 29, 18) and their fusion partners, *L. esculentum* cv. Kyoryokutoko (Lane E) and *L. chmielewskii* LA1330 (Lane C). DNA was restricted with *Bam* HI. The hybridization probe was nuclear rRNA gene, *pRR*217. Prominent bands specific to each species are marked by arrows.

However, only *pRR* 217 gave visual bands. The fusion partners always showed specific bands with the nuclear rRNA gene, *pRR* 217. All the putative somatic hybrids tested had the fragments from both partners (**Fig. 4**), as they carried 48 chromosomes (2n = 4x). RFLP analysis also revealed those somatic hybrids. Lane 29 showed same band's numbers to that of lanes 26 and 18, but the bands' position of lane 29 differed from the others. For this, one considerable reason may be due to electrophoresis. However, lanes 26 and 18 gave smooth electrophoresis pictures. The other reason may be considered as different recombination of nuclear DNA from the others occurred in somatic hybrid (lane 29) according to the positions of parental-specific bands. From RFLP analysis, it is considered that tetraploid hybrids contain two partners' fragments, only different partner protoplasts are fused, and pre-selection using IOA treatment and culture medium regulation are efficient to allow somatic hybrids develop.

This report has provided important information that will allow studies of the mechanism of embryogenesis through cell fusion in the future at histological and molecular levels. Somatic hybridization via somatic embryogenesis will also be interesting for practical breeding programs, e.g. cross-compatibility to tomato, fertility, and inheritance of introduced genes. Further experiments with somatic hybrids of *L. esculentum* and *L. chmielewskii* will open an new way to produce high soluble solid and sucrose accumulation in fruits.

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