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Original Paper

Fertile somatic hybrids between *Solanum integrifolium* and *S. sanitwongsei* (syn. *S. kurzii*) as candidates for bacterial wilt-resistant rootstock of eggplant

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Abstract UV-irradiated cotyledonary protoplasts of *Solanum integrifolium* and iodoacetamide-treated cotyledonary protoplasts of *S. sanitwongsei* were electrofused and cultured. Regenerated plants were classified into three groups based on morphology and genomic *in situ* hybridization data. Morphology of the first group was intermediate between those of parental species. The plants bore fruits with viable seeds and had a chromosome number of $2n=48$, the sum of the parental chromosome numbers, suggesting that they were symmetric fusion hybrids. Morphology of the plants in the second group was more *S. integrifolium*-like than that of the first group, and had two sets of *S. integrifolium* chromosomes and one set of *S. sanitwongsei* chromosomes. In contrast, plants in the third group had one set of *S. integrifolium* chromosomes and two sets of *S. sanitwongsei* chromosomes. Plants in the second and third groups were less vigorous than those in the first group, and bore few fruits. Electrophoretic analysis of the isozymes shikimate dehydrogenase, isocitrate dehydrogenase, and phosphoglucomutase, as well as random amplified polymorphic DNA analysis, demonstrated that 23 of regenerated plants from the three groups were somatic hybrids. The plants in the first group grew more vigorously than the parental plants and produced more than 5000 seeds per plant. The fertile somatic hybrids obtained in this study may be suitable candidates for eggplant rootstocks.

Key words: Eggplant rootstock, fertile somatic hybrids, genomic *in situ* hybridization, *Solanum integrifolium*, *Solanum sanitwongsei*.

The eggplant (*Solanum melongena*) is originated from India, and is now widely cultivated as a vegetable in tropical and temperate regions around the world (Kalloo 1993). Soil-borne diseases, such as bacterial wilt caused by *Ralstonia solanacearum*, *Fusarium* wilt caused by *Fusarium oxysporum*, and *Verticillium* wilt caused by *Verticillium dahliae*, are the major factors limiting eggplant production (Goth et al. 1991; Kalloo 1993). Breeding of resistant cultivars is an ideal method to overcome these diseases. The genetics of resistance has been extensively studied in tomato, and resistant cultivars have been bred using related wild species as sources of resistant genes (Yamakawa et al. 1978). Although some breeding efforts to produce resistant eggplants have been reported, the number of commercial resistant cultivars is limited (Goth et al. 1991; Kalloo 1993; Wang et al. 1998). Methyl bromide, a soil fumigant, was formerly used worldwide to control soil microbe populations; however, it was phased out in 2005 following the negotiation of the Montreal Protocol, because it contributes to disruption of the ozone layer (Anderson et al. 1989). Alternative agrochemicals used to suppress soil pathogens have caused health problems for farmers and environmental contamination. Moreover, controlling soil pathogens with agrochemicals is always incomplete and expensive.

Use of rootstocks is another ideal method for avoiding bacterial wilt and other soil-borne diseases, and rootstocks are extensively used for eggplant production in Japan. Among rootstock species, *S. integrifolium* is resistant to *Fusarium* wilt and remains vigorous until the later stages of eggplant production, making it the most common rootstock in Japan (Tachibana 1994). Although this species was reported to be more resistant to bacterial wilt than other eggplant cultivars (Sheela et al. 1984; Ozaki and Kimura 1989), plants on this rootstock are severely damaged by the pathogen. Another species, *S. sanitwongsei* (syn. *S. kurzii*) is highly resistant to bacterial wilt, and has recently been recognized as a rootstock, but it is not very vigorous, especially at the

Abbreviations: GISH, genomic *in situ* hybridization; RAPD, random amplified polymorphic DNA; UV, ultra-violet

early stages of eggplant production. Moreover, its thinner stem is a limiting factor for its use as a rootstock because it makes grafting more difficult. Therefore, it has been desired to produce a novel rootstock with vigorous growth and resistances to both *Fusarium* wilt and bacterial wilt by crossing these two rootstock species.

In the genus *Solanum*, McCammon and Honma (1983) succeeded in producing interspecific hybrids between *S. melongena* and *S. torvum*, but the F_1 plants were sterile. Interspecific hybrids have also been produced between *S. melongena* and *S. sisymbriifolium* using an embryo rescue technique but they died within a few days (Sharma et al. 1984). Similarly, our repeated trial of crossing failed to produce the F_1 hybrid between the two target rootstock species, *S. integrifolium* and *S. sanitwongsei*.

To overcome the difficulty in interspecific hybridization, somatic hybridization is considered to be an alternative technique for producing the interspecific hybrids and a number of somatic hybrids have previously been produced in the genus *Solanum* (Gleddie et al. 1986; Guri and Sink 1988; Daunay et al. 1993; Asao et al. 1994). In the present study, we report the successful results on the production of somatic hybrids between *S. integrifolium* and *S. sanitwongsei*, and their preliminary characters as eggplant rootstocks.

Materials and methods

Plant materials and protoplast isolation

Solanum integrifolium 'Akanasu' (Takii Seed Co., Kyoto, Japan) and *Solanum sanitwongsei* 'Karehen' (Japan Seed Trade Association, Tokyo, Japan) were used in this study. *In vitro*-sown seedlings were obtained as described in Iwamoto (1999). Protoplasts were isolated and purified using the method described by Iwamoto and Ezura (2006).

Inactivation treatment of protoplasts

Pelleted *S. sanitwongsei* protoplasts were resuspended in 0.2 mM iodoacetamide (pH 5.8) containing 0.4 M mannitol and 2.5 mM $CaCl_2$ and then incubated at 25°C for 10 min. After iodoacetamide-treatment, the protoplasts were centrifuged at 100 g for 3 min and washed once in 0.4 M mannitol supplemented with 2.5 mM $CaCl_2$. Protoplasts of *S. integrifolium* were suspended in 0.4 M mannitol supplemented with 2.5 mM $CaCl_2$ and then irradiated with ultra-violet (UV) light from a distance of 30 cm for 10 s using a GL-15 lamp (National Co., Osaka, Japan). They were then suspended in the same solution at a density of 5×10^5 protoplasts ml^{-1} and kept on ice.

Electrofusion

Prior to fusion, the inactivated cotyledonary protoplasts of both species were mixed at a 1:1 ratio, 0.75 ml aliquot was pipetted into a 15×60 mm Petri dish, and an immersible multi-electrode (FTC-33D5; Shimadzu, Kyoto, Japan) was placed in the aliquot. The protoplasts were aligned by a 1 MHz alternating current at 150 V/cm for 30 s and then fused by three direct current square pulses at 750 V/cm for 20 μs using a somatic hybridizer (SSH-1; Shimadzu, Kyoto, Japan).

Protoplast culture and plant regeneration

The protoplast culture protocol followed the method of Iwamoto and Ezura (2006). One thousand green calli were used for plant regeneration. The rooted plants were acclimatized and cultivated in a greenhouse according to the method of Iwamoto (1999).

Field trials and morphological observation

To study the fertility and field performance of the somatic hybrids, 24 hybrid clones and 2 parental lines were propagated by stem cuttings. Rooted cuttings 20–25 cm tall were transplanted in a field at the Agricultural, Food, and Environmental Sciences Research Center of Osaka Prefecture, Habikino, Japan in April 1998, and cultivated until November 1998. Five plants were used for each genotype. Culture practices, fertilization, weed control, and fungicide and insecticide treatments were similar to those used in commercial eggplant production. Five plants of each genotype were evaluated for height, stem diameter, leaf shape, petal color, flower number per inflorescence, and root dry weight. Pollen fertility was determined by staining a minimum of 200 grains from each plant with 1% (w/v) acetocarmine. The mean value for pollen stainability was estimated in September. Fruit productivity was examined in November, and the number of ripe and unripe fruits, fruit diameter, and fruit moisture content were recorded. The number of seeds per fruit was calculated from a sample of 20 fruits.

Isozyme analysis

The electrophoretic patterns of the isozymes of shikimate dehydrogenase (SKDH; EC 1.1.1.25), phosphoglucomutase (PGM; EC 2.7.5.1), and isocitrate dehydrogenase (IDH; EC 1.1.1.41) were examined using 200 mg of young leaves from *in vitro*-grown plants. After homogenization of the tissues in 1 ml of extraction buffer and centrifugation, the supernatant was used for polyacrylamide slab gel electrophoresis (Hirai et al. 1986), and the gels were stained according to Wendel and Parks (1982).

DNA extraction and random amplified polymorphic DNA (RAPD) analysis

Total DNA was extracted as described in Hashizume et al. (1996). In total, 57 polymerase chain reaction (PCR) primers were used to reveal the polymorphisms between the parents. Primers positive for each polymorphism were used to identify hybrids. The 12-mer primers were originally synthesized as arbitrary primers by standard phosphoramidite chemistry on a DNA synthesizer (Model 392; Applied Biosystems Inc., CA, USA). Following amplification, the PCR products were loaded in 1.5% (w/v) agarose gels containing 1 $\mu g\,ml^{-1}$ ethidium bromide in 1×TBE buffer, run at 100 V for about 30–40 min, and then photographed under UV light with Polaroid film.

Lambda DNA digested with *EcoR*I and *Hind*III was used as a molecular marker.

Chromosome counting and genomic in situ hybridization

Chromosome preparations were made by the enzymatic maceration/air drying method (Fukui and Iijima 1991). They were spread onto glass slides and stained with 4% Giemsa solution. DNA denaturation, *in situ* hybridization, and detection/amplification were performed using a previously described method (Fukui et al. 1994; Escalante et al. 1998).

Results

The fused products underwent their first cell divisions after 4 to 5 days of protoplast culture, and some of the cells developed into colonies (Figure 1A and B). Green shoots were regenerated in 792 of 1000 calli 2 to 3 weeks after transfer onto shoot regeneration medium (Figure 1C). The regenerated parental plants were removed based on their morphological characters, and 24 putative somatic hybrids were selected (Table 1). Twenty-three of the 24 putative somatic hybrids grew normally and were used for further analysis, whereas remaining one plant showed severe chlorosis and was discarded.

The selected 23 putative somatic hybrids were classified into three groups based on their morphologies (Table 2). The morphology of the first group, which included 13 plants, was intermediate between the parents in terms of its leaves (Figure 2A), flower-buds (Figure 2B), flower color (Figure 2C), and immature and mature fruits (Figure 2D, Table 2). The stem of this group of hybrid was thicker than that of *S. sanitwongsei*, and the plants were more vigorous than their parents and bore fruits well (Figure 2E, Table 2). Indeed, more than 10 seeds were obtained from a single fruit (Figure 2F, Table 2), and each plant produced more than 5000 seeds (about 50 ml) (Table 2). The chromosome number was $2n=48$, which is the sum of its parents (Table 2). No apparent morphological diversity was found within this group.

The morphology of the second group including 4 plants was more *S. integrifolium*-like than that of the first group (Table 2), in terms of the leaves (Figure 3A), inflorescences (Figure 3B), and mature fruits (Figure 3C). The flower color and moisture content of the fruit from the second group closely resembled that of *S. integrifolium* (Table 2). On the other hand, the morphology of the third group, which included 6 plants, was more *S. sanitwongsei*-like than that of the first group (Figure 3, Table 2). All the ten plants involved in the second and third groups were also less vigorous, and bore small numbers of fruit that contained either no seeds or only one occasional seed. Furthermore, the plants had malformed leaves with slight chlorosis.

Isozyme analysis of the regenerated plants showed that 23 regenerated plants in the three groups were hybrids with parent-specific bands for shikimate dehydrogenase (Figure 4), isocitrate dehydrogenase, and

Table 1. The results of somatic hybridization between *Solanum integrifolium* and *S. sanitwongsei*.

Total number of calli[1]	Number of regenerated calli[2]	Identification of regenerated plant[3]		
		S. integrifolium	Somatic hybrid	*S. sanitwongsei*
1000	792	336	24	428

[1] Total number of calli plated on regeneration medium, 5–6 weeks after protoplast fusion.
[2] Regenerated calli counted 16 weeks after protoplast fusion.
[3] Identification of somatic hybrid plants and their parents was based on the morphological analysis of greenhouse-grown plants.

Table 2. Characteristics of *S. integrifolium*, *S. sanitwongsei* and three groups of somatic hybrids.

	S. integrifolium	Somatic hybrids			*S. sanitwongsei*
		group 2	group 1	group 3	
Height (cm)	118.6±9.7	136.4±22.3	203.4±12.3	136.0±26.5	155.2±20.1
Stem diameter (cm)	2.33±0.44	2.37±0.40	4.91±0.81	2.00±0.53	2.91±0.57
Leaf shape	Lobed	Malformed	Slightly divided	Malformed	Deeply divided
Petal color	White	White	Light purple	Purple	Purple
Stripe color	Red purple	Red purple	Red purple	White	White
Mean number of flower per inflorescence	9.6±2.4	9.2±2.8	13.0±1.6	5.6±0.8	2.7±0.2
Mean diameter of fruit (cm)	51.3±3.0	23.5±1.6	19.1±1.8	13.5±1.1	10.4±1.2
Mean number of fruits per plant	77.0±8.2	29.6±5.1	481.6±92.6	38.0±16.6	930.0±136.0
Mean number of seeds per fruit	349.3±34.3	0.6±0.5	16.8± 2.3	0.0±0.0	34.9±4.4
Moisture content of fruit	82.9±1.8	81.6±1.9	81.5±0.8	69.7±3.4	69.3±3.6
Mean dry weight of root per plant (g)	29.2±4.0	18.4±6.2	41.3±7.8	28.6±4.4	37.3±5.6
Mean volume of seeds per plant (ml)	256.4±54.6	0.0±0.0	49.6±9.6	0.0±0.0	186.2±31.9
Pollen viability (%)	92.9±1.8	52.9±1.6	78.8±2.1	31.1±7.3	94.3±0.8
Chromosome number	24	67, 70, 72	48	72	24
Number of plants		4	13	6	

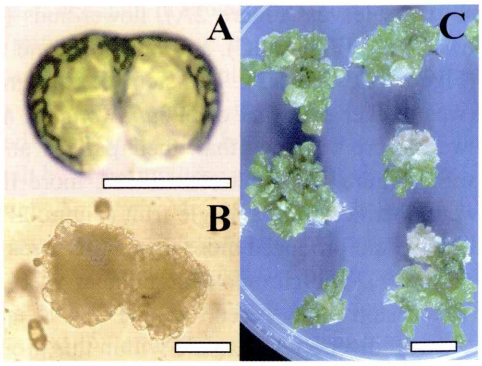

Figure 1. Production of somatic hybrids between *Solanum integrifolium* and *S. sanitwongsei*. (A) Fused cell. Bar=30 μm. (B) Colony formation after 3 weeks of culture. Bar=200 μm. (C) Shoots regenerated from calli 3 weeks after transfer onto shoot regeneration medium. Bar=1 cm.

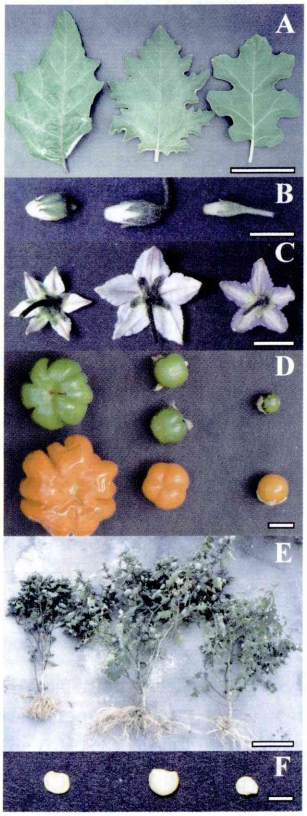

Figure 2. Plant morphology. Illustrated samples in A–E were *S. integrifolium* (left), the amphidiploid somatic hybrid of first group (center), and *S. sanitwongsei* (right). (A) Leaf. Bar=10 cm; (B) Flower-bud. Bar=1 cm; (C) Flower color. Bar=1 cm; (D) Immature fruit (upper) and mature fruit (lower). Bar=10 cm; (E) Entire plant. Bar=50 cm; (F) Seed. Bar=4 mm.

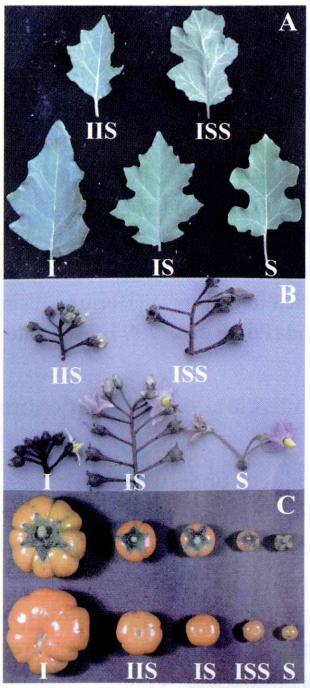

Figure 3. Plant Morphology. Illustrated samples in A–C were *S. integrifolium* (I), hexaploid somatic hybrid of second group (IIS), the amphidiploid somatic hybrid of first group (IS), hexaploid somatic hybrid of third group (ISS), and *S. sanitwongsei* (S). (A) Leaf shape; (B) Inflorescence; (C) mature fruit.

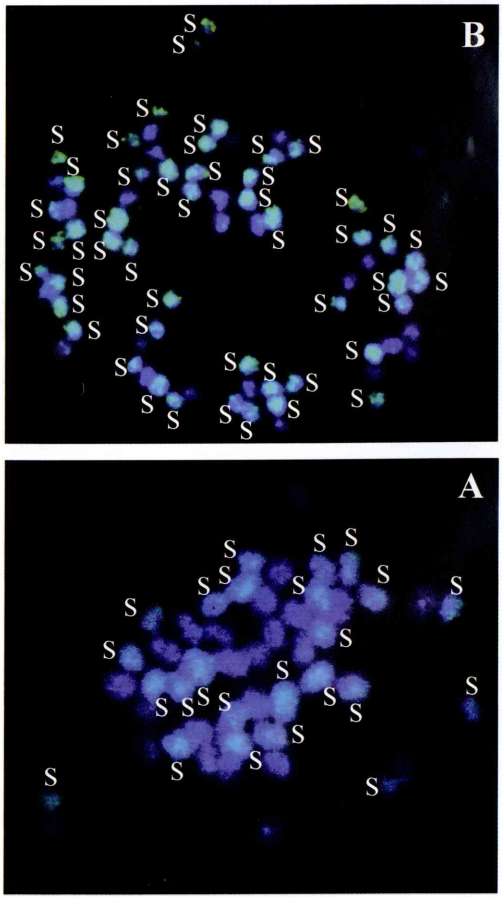

Figure 6. Identification of parental chromosomes in somatic hybrid using genomic *in situ* hybridization. (A) Somatic metaphase chromosomes of amphidiploid somatic hybrid ($2n=4x=48$) with 24 chromosomes of *S. sanitwongsei* (Green; S) and 24 chromosomes of *S. integrifolium* (Blue). (B) Somatic metaphase chromosomes of hexaploid somatic hybrid ($2n=6x=72$) showing 48 chromosomes of *S. sanitwongsei* (Green; S) and 24 chromosomes of *S. integrifolium* (Blue).

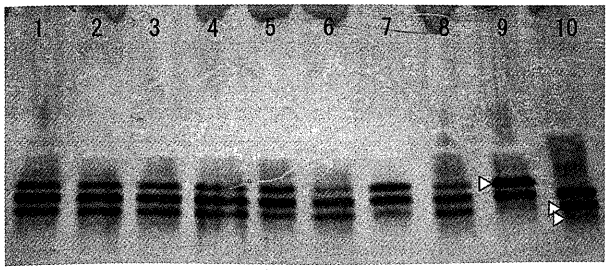

Figure 4. Shikimate dehydrogenase (SKDH) banding patterns of *S. integrifolium*, *S. sanitwongsei*, and the somatic hybrids. Lane 1–7: somatic hybrids. 8: the mixture of parental extracts. 9: *S. integrifolium*, 10: *S. sanitwongsei*. Parent-specific fragments are indicated by arrowheads (▷).

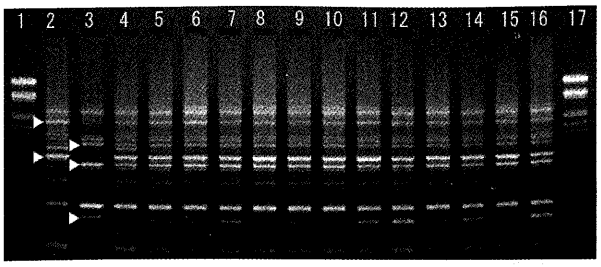

Figure 5. Amplification of genomic DNA by random primer RA12–28. Lane 1,17: Molecular maker. 2: *S. integrifolium*. 3: *S. sanitwongsei*. 4: Mixture of parental DNA. 5–16: somatic hybrids. Parent-specific polymorphic fragments are indicated by arrowheads (▷).

phosphoglucomutase.

In total, 57 random primers were used to survey the polymorphisms between the parents. Among them, 20 polymorphic primers were used for analysis of the regenerated plants, and 43 *S. integrifolium*-specific and 57 *S. sanitwongsei*-specific bands were found in each regenerated plant (n=23) in the three groups. Because all of the RAPD markers from the parental lines were found in all the 3 hybrid groups, the majority of the two genomes were retained in 23 somatic hybrids (Figure 5). Genetic diversity within the first group was not observed by RAPD.

The karyotypes of *S. integrifolium* and *S. sanitwongsei* are very similar, so most of the chromosomes in the somatic hybrids could not be discriminated through routine cytological procedures. Instead, GISH was used to reveal the genomic compositions of the somatic hybrids. GISH analysis of the 13 somatic hybrids from the first group that possessed 48 chromosomes revealed that 24 *S. sanitwongsei* chromosomes were present in all of the hybrids (Figure 6A). Consequently, these hybrids were confirmed as amphidiploid plants ($2n$=$4x$=48), indicating that these hybrids were produced by symmetric fusion.

Two of four plants in the second group had 72 chromosomes ($2n$=$6x$=72). GISH analysis showed that 24 of the 72 chromosomes were from *S. sanitwongsei*, while the remaining 48 were from *S. integrifolium*. The second group should therefore be called asymmetric hybrids. The morphological and GISH results strongly suggest that the plants in the second group have two sets of *S. integrifolium* chromosomes and one set of *S. sanitwongsei* chromosomes. Remaining 2 plants of the second group had 67 and 70 chromosomes, respectively.

Six plants in the third group had 72 chromosomes ($2n$=$6x$=72), and 48 were identified by GISH as chromosomes from *S. sanitwongsei*, while the remaining 24 were from *S. integrifolium*. Plants in the third group should therefore also be called asymmetric hybrids. The morphological and GISH results strongly suggest that the plants in the third group have one set of *S. integrifolium* chromosomes and two sets of *S. sanitwongsei* chromosomes ($2n$=$6x$=72, Figure 6B).

Discussion

Somatic hybridization was first reported by Carlson et al. (1972). Subsequently, a number of studies have examined cell fusion; however, only a few studies have reported the successful use of somatic hybrids for further breeding of commercially valuable cultivars. Since somatic hybrids are usually produced between relatively remote parental species, one major difficulty is sterility of the resultant hybrids that prevents backcrossing. In this study, however, the somatic hybrids in the first group had fertile pollen and produced fruit and seed quantities comparable to those of their parental species. Therefore, this type of the hybrid may be used as a rootstock in eggplant production, because both parents are already used as rootstocks.

Use of rootstocks is one possible strategy to overcome vulnerability to soil-borne diseases in fruit and fruit-vegetable production. In the case of fruit-vegetables, rootstocks have already been extensively used for tomato, eggplant, watermelon, and melon production in Japan. Breeding of rootstock cultivars is easier and more time efficient than scion cultivars, because the object of breeding is limited to such factors as resistance to soil-borne diseases, growth-supporting traits, and graft compatibility. Breeders can also disregard fruit characters, which are the most complicated and important characters. Although a number of somatic hybrids between eggplant and related species have been reported (Gleddie et al. 1986; Guri and Sink 1988; Daunay et al. 1993), they may have many characters undesirable for commercial eggplant cultivars. Utilization of somatic hybrids for rootstock cultivars is one of the best ways to avoid this problem.

The somatic hybrids presented in this study provide an example of the use of cell fusion techniques for breeding commercial cultivars. Because rootstock cultivars are propagated by seed, production of viable seeds is the key

to using rootstocks in agricultural production. In this study, the plants in the first group, which were symmetric fusion products, bore fruit with viable seeds by natural self-pollination. If their inbred progeny is genetically uniform, they may be directly used as rootstocks for eggplant production. Productivity, resistance to soil-borne diseases, graft compatibility, and other important characteristics of rootstocks should be further examined in a field trial.

The plants in the other groups obtained in the present study should be asymmetric products. Between the two groups of the hexaploid plants, genome composition was correlated with morphological characters. Although no parent-specific RAPD bands were lost in the second group, some of the chromosomes were lost in two somatic hybrids. Thus, more detailed study is required to clarify the chromosome constitution of this group.

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