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Genetic analysis of stunted growth by nuclear-cytoplasmic interaction in interspecific hybrids of *Capsicum* by using RAPD markers

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Abstract. When eight cultivars of *Capsicum annuum* were used as female parents in interspecific crosses with two accessions of *C. chinense*, dwarfism occurred in hybrids originating from 10 out of 16 combinations, while hybrids of the remaining 6 combinations grew normally. In contrast, when *C. chinense* was used as female parent, all of the hybrids showed severely stunted growth as if affected by a virus. These results suggested that the stunted growth expressed in the cross of *C. chinense* × *C. annuum* is caused by an interaction between nuclear gene(s) from *C. annuum* and the cytoplasm of *C. chinense*. To examine the number of nuclear gene(s) which cause(s) the stunted growth, we backcrossed F_1 hybrids of *C. annuum* × *C. chinense* to *C. chinense*. About one-quarter of the progeny in the backcrossed hybrids of *C. chinense* × (*C. annuum* × *C. chinense*) showed the same stunted growth shown by the F_1 hybrids of *C. chinense* × *C. annuum*, suggesting that two complementary genes of *C. annuum* cause the stunted growth. However, the higher abortion rates of ovules and lower germination percentage of seeds in *C. chinense* × *C. annuum* than in the selfed *C. chinense* implied that the genetic ratio of the stunted type would have been higher than that observed in the *C. chinense* × (*C. annuum* × *C. chinense*) progeny. We then attempted a linkage analysis between the stunted growth and randomly amplified polymorphic DNA (RAPD) of *C. chinense* × (*C. annuum* × *C. chinense*) progeny. A RAPD marker that associated with 94% of the stunted plants but not with 94% of the normal one was identified. This confirmed that a single nuclear gene of *C. annuum* which is linked to the RAPD marker with a recombination value of 6% causes the stunted growth in an interaction with the cytoplasm of *C. chinense*.

Key words: *Capsicum* – Interspecific cross – Stunted growth – Nuclear-cytoplasmic incompatibility – RAPD makers

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Introduction

Interspecific crosses are an effective means by which to incorporate agronomically important traits from related species into cultivars. In the genus *Capsicum*, *C. chinense* is known to be a donor for several desirable traits such as TMV resistance and multiple fruits per node (Subramanya 1983). The transfer of these *C. chinense* traits into *C. annuum* could possibly result in reduced harvest costs and increased yield. However, this approach often encounters such interspecific hybridization barriers as embryo and/or endosperm abortion and hybrid weakness. So far, two types of barriers have been observed in the interspecific hybridization of *Capsicum* spp. the first type dwarfism, has been found in interspecific hybrids and is characterized by the termination of leaf emergence after the bearing of several normal leaves. This dwarfism is caused by two complementary dominant genes, with the *AAbb* genotype being possessed by the *C. annuum* part, and the *aaBB* genotype by *C. chinense* and *C. frutescens* (Yazawa et al. 1989, 1990). The second type, abnormal growth characterized by female-sterility and true leaves that become shrunken as if affected by virus, was found in interspecific hybrids of *C. frutescens* × *C. baccatum* (Pickersgill 1971) and *C. chinense* × *C. baccatum* (Yazawa et al. 1990). This type of abnormal growth has been named the virus-like syndrome (VLS). Segregation data from backcrosses of normal F_1 plants to both parents have revealed that VLS is due to an interaction between the cytoplasm of *C. chinense* and a nuclear gene from *C. baccatum* (González de León 1986). However, little has been known about the occurrence of a similar phenomenon in other interspecific hybrids of *Capsicum* spp.

Stunted plants, whose phenomenon was named "stunted growth" in this study, were expressed in the cross of *C. chinense* × *C. annuum*, and dwarf plants were expressed in the cross of *C. annuum* × *C. chinense*. This study was conducted to clarify the genetic mechanism for the expression of stunted growth. We used randomly amplified polymorphic DNA (RAPD) (Martin et al. 1991) to find a DNA marker for a nuclear gene in *C. annuum* responsible for the stunted growth trait.

Materials and methods

Plant materials

Eight cultivars of *Capsicum annuum* L., 'Oh-natsume', "Enken-amanaga', 'Zairai-amanaga', 'Takanotsume', 'Yatsubusa', 'Fushimi-amanaga', 'Shishito' and 'Shosuke', and *C. chinense* Jacq. Plant Introduction (PI) 159236 and PI 315008 were grown in the greenhouse. Reciprocal crosses between *C. annuum* and *C. chinense* were made. One F_1 hybrid plant, 'Oh-natsume' × PI 159236 was selfed and backcrossed to both parents in order to obtain B_1F_1s. These B_1F_1 and F_2 seeds were sown to determine the segregation ratio of normal and stunted plants.

Isolation of genomic DNA and polymerase chain reaction (PCR)

Total DNAs were extracted from young leaves of the plants grown in the greenhouse according to the method of Rogers and Bendich (1985). Polymerase chain reactions (PCR) were conducted in a microtube containing a 25-μl aliquot comprised of 2.5 μl 10 × PCR buffer containing 100 m*M* TRIS-HCl, 15 m*M* $MgCl_2$, 800 m*M* KCl, 5 mg/ml BSA, 1% (w/v) Na-cholate and 1% (w/v) Triton X-100, 1.25 m*M* each of dNTP (Pharmacia), 0.5 ng of template DNA and 2 units of T*th* polymerase (TOYOBO Co, Osaka, Japan). PCR were performed in a DNA thermal cycler (ASTEC/PC-700) for 43 cycles of denaturation at 94 °C (1min), annealing at 51 °C (1 min) and extension at 72 °C (2 min). Initial denaturation was conducted for 2 min at 94 °C. Seven primers were used: SSU-1F, SSU-2F, SSU-1R, SSU-2R, SSU-3R (Omura et al. 1991), KIN4 and KIN8. Primer SSU-2F, which produced a RAPD marker for the stunted growth, had the following sequence: 5'-ATGTGGAAGCTGCCCATGTTC-3'.

Gel electrophoresis and Southern hybridization

RAPD products were visualized with ethidium bromide after electrophoresis on 1.5% (w/v) agarose gels. For more detailed analysis, RAPD products were electrophoresed on agarose gels and then transferred to a nylon membrane (Hybond N^+, Amersham, UK). The selected RAPD fragment to be used as a probe was excised from the agarose gels and recovered according to the Geneclean II Kit protocol (BIO 101, USA). Labelling with ECL system (Amersham, UK), hybridization and detection of the hybridized probe were according to the manufacturer's protocol.

Results

Characterization of interspecific hybrids

Many seeds were obtained in the cross of *C. annuum* as female parent and *C. chinense* as male parent. Of these, seeds with a brownish discolored embryo and/or endosperm were found to be inviable. The F_1 plants

Table 1. Characteristics of interspecific hybrids between eight cultivars of *Capsicum annuum* and two accessions of *C. chinense*. APH and ANL were measured 2 months after sowing

C. annuum	PI 159236			PI 315008		
♀	APH	ANL	Phenotype	APH	ANL	Phenotype
Oh-natsume	15.5	9.6	Normal	14.6	10.6	Normal
Enken-amanaga	27.2	13.8	Normal	17.1	11.5	Normal
Zairai-amanaga	21.8	11.7	Normal	21.8	11.7	Normal
Takanotsume	7.5	6.0	Dwarf	10.6	8.0	Dwarf
Yatsubusa	7.0	4.7	Dwarf	8.0	8.0	Dwarf
Fushimi-amanaga	5.0	3.5	Dwarf	9.7	9.4	Dwarf
Shishito	7.4	4.5	Dwarf	8.4	5.0	Dwarf
Shosuke	4.8	6.1	Dwarf	5.2	5.4	Dwarf
♂	APH	ANL	Phenotype	APH	ANL	Phenotype
Oh-natsume	2.0	0.7	Stunted	2.1	4.0	Stunted
Enken-amanaga	3.8	3.4	Stunted	1.3	2.0	Stunted
Zairai-amanaga	3.6	3.0	Stunted	3.4	3.8	Stunted
Takanotsume	3.8	4.3	Stunted	3.6	3.8	Stunted
Yatsubusa	2.4	4.0	Stunted	3.2	3.0	Stunted
Fushimi-amanaga	5.9	4.5	Stunted	7.1	5.0	Stunted
Shishito	5.4	4.5	Stunted	4.7	2.0	Stunted
Shosuke	4.3	4.3	Stunted	3.6	3.8	Stunted

APH, Average plant height (cm); ANL, average number of leaves

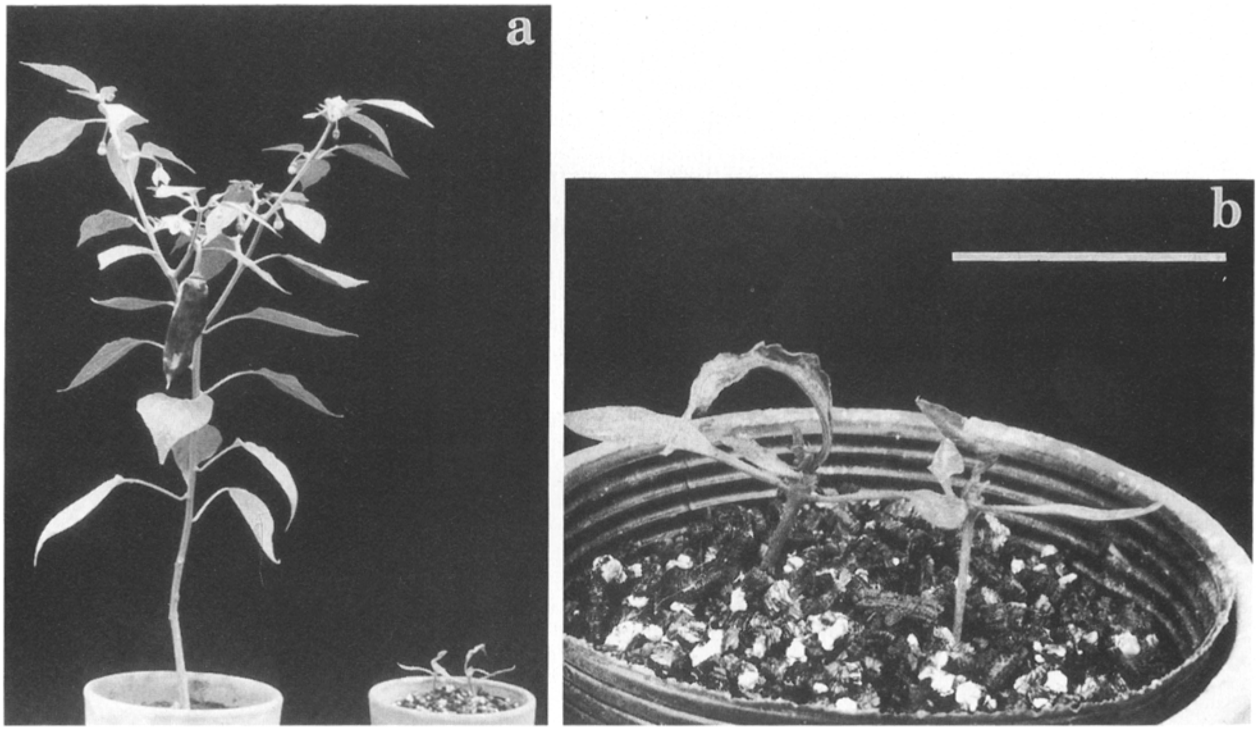

Fig. 1a, b. Developmental differences 4 months after sowing in reciprocal F_1 hybrids between *C. annuum* 'Oh-natsume' and *C. chinense* PI 159236. **a** The hybrid of *C. annuum* × *C. chinense* (*left*) grows normally; the hybrid of *C. chinense* × *C. annuum* (*right*) exhibits stunted growth. **b** The hybrid of *C. chinense* × *C. annuum*. *Bar*: 5 cm

showed either dwarf or normal morphology depending on cultivars of *C. annuum* (Table 1). When 'Takano-tsume', 'Yatsubusa', 'Fushimi-amanaga', 'Shishito' and 'Shosuke' were used as the female parent, the F_1 hybrids showed the dwarf morphology. These plants stopped expanding new leaves and failed to bear flower buds after they had borne five to six leaves. These characteristics are similar to those reported by Yazawa et al. (1989). On the other hand, when 'Oh-natsume', 'Enken-amanaga' and 'Zairai-amanaga' were used as the female parent, the F_1 hybrids grew normally and produced fertile pollen.

In the cross of *C. chinense* as female parent and *C. annuum* as male parent, seed yield was much lower. A small number of F_1 hybrids obtained in all combinations showed the stunted growth morphology (Table 1). After these hybrids had expanded their cotyledons, they showed severely shortened or compressed internodes and the loss of apical dominance, their lamina was narrow, asymmetric, thickened and shrunken as if affected by virus (Fig. 1a, b).

Appearance of plants with stunted growth in the F_2 and backcrossed progeny

Out of the 6 hybrids of *C. annuum* × *C. chinense* that grew normally, acetocarmine staining of pollen grains revealed that 'Oh-natsume' × PI 159236 had the highest pollen fertility. 'Oh-natsume' × PI 159236 was then reciprocally backcrossed to both 'Oh-natsume' ('ON') and PI 159236 (PI) and selfed. Of these crosses, only 'ON' × ('ON' × PI), ('ON' × PI) × 'ON', ('ON' × PI) × PI and the F_2 produced normal plants. In the progeny of PI × ('ON' × PI), 279 normal and 79 stunted plants appeared, with a segregation ratio of 3:1 ($0.1 < P < 0.25$) (Table 2, Fig. 2). However, there was a possibility that embryo abortion and/or failure of endosperm

Table 2. Segregation and their average plant height in F_1s, B_1F_1s and F_2 between *C. annuum* and *C. chinense*. Plant height were measured 2 months after sowing

Crosses	Number of plants and their average plant height (cm)		Total
	Normal (cm)	Stunted (cm)	
ON × PI	15 (19.9)	0	15
PI × ON	0	15 (3.9)	15
(ON × PI) × ON	151 (16.6)	0	151
(ON × PI) × PI	97 (16.7)	0	97
ON × (ON × PI)	47 (19.6)	0	47
PI × (ON × PI)	279 (16.5)	79 (5.3)	358
(ON × PI) self	100 (17.3)	0	100

ON, *C. annuum* cv 'Oh-natsume'; PI, *C. chinense* 'PI' 159236'

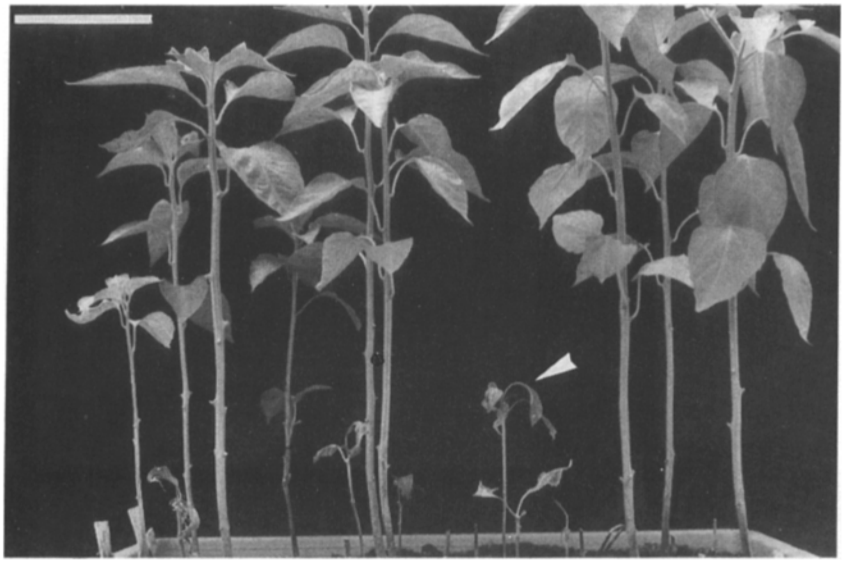

Fig. 2. Segregation of normal and stunted plants in PI × ('ON' × PI) progeny. *Arrowhead* indicates 1 of stunted plants. *Bar*: 5 cm

development in the progeny affected the segregation ratio. Therefore, we counted the number of aborted seeds at early and later stages as well as the number of seeds with a hard seed coat and their germination percentage (Table 3). Some ovules were found to be aborted at early or later stages after fertilization and a lower germination percentage than that in the selfed PI was observed in the cross of PI × ('ON' × PI).

Detection of a RAPD marker linked to nuclear gene(s) for stunted growth

To detect RAPD markers linked to *C. annuum* nuclear gene(s) associated with the stunted growth, we first screened those primers showing different band patterns between PI and 'ON'. Of the seven primers surveyed six showed polymorphism between PI and 'ON' (Fig. 3). Approximately 88 PCR products ranging from 300 to 2800 base pairs (bp) were totally amplified in both parents by these six primers, giving an average of 14.6 products per primer. Of these, 45 products were specific to each parent, with an average of 7.5 products per primer; 21 products were specific to 'ON', with an average of 3.5 products per primer.

To confirm whether any of these products were linked to putative stunted growth-related gene(s) in 'ON', we analyzed a total of 68 plants from the PI × ('ON' × PI) population consisting of 34 stunted and 34 normal plants. A 610-bp fragment amplified by SSU-2F was detected in 'ON', the reciprocal F_1s, 32 stunted and 2 normal plants, but not in PI, 32 normal and 2 stunted plants (Fig. 4a). The marker product of 610 bp was also found in the other cultivars of *C. annuum* used in this study except for 'Takanotsume' and 'Yatsubusa' (Fig. 5).

Hybridization analysis was performed to confirm whether or not the 610-bp fragment detected in 'ON' was homologous to that found in the reciprocal F_1s and most of the stunted plants in the PI × ('ON' × PI) cross. RAPD products obtained in 'ON', PI, the reciprocal F_1s and PI × ('ON' × PI) progeny using SSU-2F were separated on agarose gels, blotted onto a nylon

Table 3. Frequencies of aborted ovules and seeds obtained in *C. chinense* (PI), *C. annuum* ('ON'), their F_1s and B_1F_1. Aborted ovules were classified into two groups according to their sizes. Percentages are shown in parentheses

Parents or crosses	Number of fruits tested	Number of aborted ovules/fruit		Number of obtained seeds/fruit	Total	Germination percentage of seeds
		< 0.5 mm	> 0.5 mm			
PI self	6	26.2[a] (22.1)	14.5 (12.3)	77.5 (65.6)	118.2	96.4
ON self	6	12.7 (4.3)	0.5 (0.2)	281.0 (95.6)	294.2	88.4
ON × PI	6	38.2 (16.0)	2.0 (0.8)	200.6 (83.2)	240.8	14.3
PI × ON	6	38.3 (24.7)	76.8 (49.6)	39.8 (25.7)	155.0	4.2
PI × (ON × PI)	24	4.3 (14.5)	4.9 (16.4)	20.5 (69.1)	29.6	72.9

[a] Average number of aborted ovules and obtained seeds per fruit

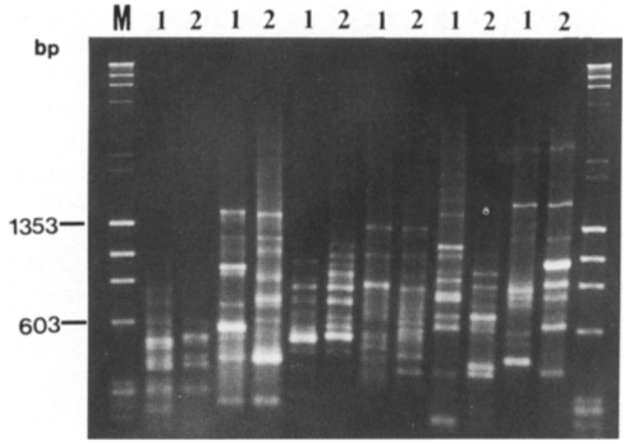

Fig. 3. Electrophoretic patterns of PAPD products obtained with each of primers, KIN4, KIN8, SSU-2R, SSU-3R, SSU-1F and SSU-2F (*left to right*). *Lane M* is a mixture of molecular markers, i.e., ϕX174 digested with *Hae*III and λDNA digested with *Hin*dIII, *lanes 1* and *2* are parents PI and 'ON', respectively

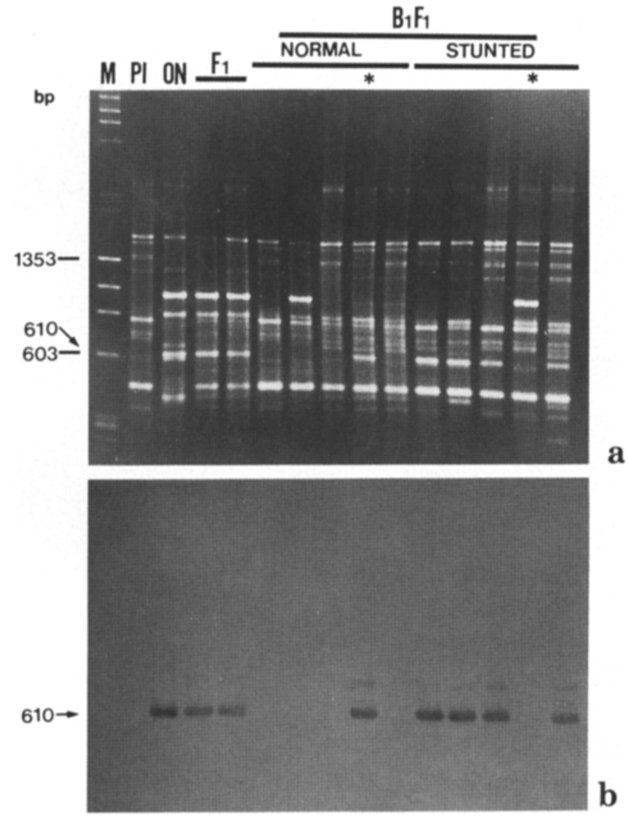

Fig. 4a, b. Electrophoretic patterns of RAPD products obtained with SSU-2F as a primer (**a**) and Southern hybridization of the 610-bp RAPD fragment detected in 'ON' (**b**). *Lane M* is a mixture of molecular markers, i.e., ϕX174 digested with *Hae*III and λDNA digested with *Hin*dIII, *lanes 1* and *2* are parents PI and 'ON', respectively, *lanes 3* and *4* are PI × 'ON' and 'ON' × PI, respectively, *lanes 5–9* and *10–14* are normal and stunted plants in PI × ('ON' × PI) progeny, respectively. Recombinant individuals are denoted by an *astersik*. **a** Ethidium bromide-stained gel. **b** The marker of 610 bp in 'ON' Southern-hybridized to the RAPDs shown in **a**. *Arrow* indicates the 610-bp marker linked to nuclear gene for stunted growth

membrane and hybridized with the 610-bp fragment recovered from RAPD products in 'ON'. The 610-bp fragment obtained in the reciprocal F_1s and the stunted plants from the PI × ('ON' × PI) cross was hybridized with that in 'ON' (Fig. 4b). No signals were detected in PI and in normal plants from the PI × (ON × PI) cross.

Discussion

In this study, two types of barriers have been shown in the interspecific hybridization between *C. annuum* and *C. chinense*, namely, the appearance of dwarf hybrids in a number of the *C. annuum* × *C. chinense* crosses and the occurrence of the stunted growth morphology in hybrids from all possible crosses of *C. chinense* × *C. annuum*.

The expression of dwarfism in interspecific hybrids seems to be caused by two complementary dominant genes (Yazawa et al. 1989). According to this theory, the *C. chinense* PI used in this study may possess one of the complementary genes; *C. annuum* 'Takanotsume', 'Yatsubusa', 'Fushimi-amanaga', 'Shishito' and 'Shosuke' may possess the other complementary gene, while 'Ohnatsume' ('ON'), 'Enken-amanaga' and 'Zairaiamanaga' do not. Interestingly, these dwarf F_1 hybrids developed new leaves again and produced flower buds after a high temperature (25°–30 °C) treatment (data not shown). Segregation of this type in the F_2 population of these hybrids is now under investigation to clarify the genetic mechanism for the appearance of dwarf hybrids.

The expression of stunted growth seems to be caused by an interaction between nuclear gene(s) from *C. annuum* and the cytoplasm of *C. chinense* because the stunted growth only occurred in those crosses where *C. chinense* was the female parent, and not in the crosses of *C. annuum* × *C. chinense*. This results indicate that nuclear gene(s) from *C. annuum* were dominantly expressed in the cytoplasm of *C. chinense*. The growth of stunted plants in the F_1 and in PI × ('ON' × PI) was not re-initiated by the high temperature treatment (data not shown).

The segregation of normal to stunted plants fitted a 3:1 ratio ($0.1 < P < 0.25$) in the PI × ('ON' × PI) progeny. However, many ovules aborted at early or later stages and the germinability of seeds was low in the cross of PI × ('ON' × PI). The data in Table 3 indicates that the difference in the number of aborted ovules over 0.5 mm in diameter in the cross of PI × 'ON' and selfed PI is significant while that in the

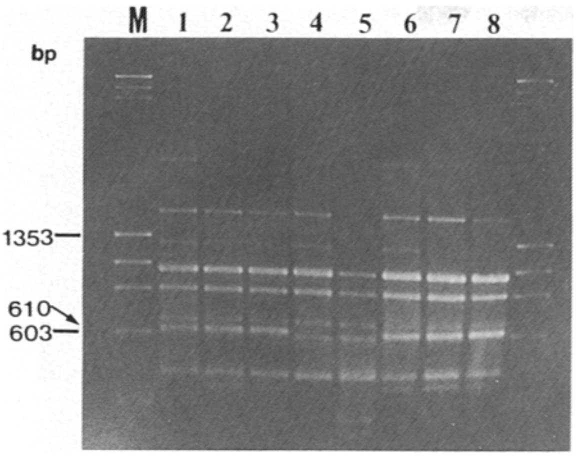

Fig. 5. Electrophoretic patterns of RAPD products obtained with SSU-2F as a primer in eight cultivars of *C. annuum*. *Lane M* is a mixture of molecular markers, i.e., ϕX174 digested with *Hae*III and λDNA digested with *Hin*dIII, *lanes 1–8* are 'Oh-natsume', 'Enken-amanaga', 'Zairai-amanaga', 'Takanotsume', 'Yatsubusa', 'Fushimi-amanaga', 'Shishito' and 'Shosuke', respectively. *Arrow* indicates the 610-bp marker linked to the nuclear gene for stunted growth

number of aborted less than 0.5 mm in diatmeter ovules is not. The percentage of aborted (over 0.5 mm) ovules was about 50% in the cross of PI × 'ON'. In addition, the germination percentage in the hybrids of PI × 'ON' was less than that in the selfed PI plants. Pickersgill (1991) reported that many interspecific crosses in *Capsicum* produce seeds incapable of normal germination because their endosperm and/or embryo does not develop properly. Therefore, the segregation ratio of the stunted type to normal plants from the cross of PI × ('ON' × PI) might be affected by the high abortion rate of ovules and low germination percentage of seeds of the stunted type and be deviated from a true segregation ratio.

In an attempt to evaluate the number of nuclear gene(s) responsible for the stunted growth morphology, the PI × ('ON' × PI) progeny were analyzed using RAPD markers. A 610-bp fragment amplified by SSU-2F appeared in 32 of the 34 stunted plants from the PI × ('ON' × PI) cross, but not in 32 of the 34 normal plants. The 1:1 segregation ratio of this marker provides evidence that a single nuclear gene of *C. annuum* causes the stunted growth. Southern hybridization also confirmed that the 610-bp fragment descended from 'ON' to the reciprocal F_1s and stunted plants in the PI × ('ON' × PI) cross. Thus, we conclude that the stunted growth morphology is caused by an interaction between a dominant nuclear gene from *C. annuum* and the cytoplasm of *C. chinense*. The role of a nuclear gene of *C. annuum* in the cytoplasm of *C. chinense* would ressemble that of *C. baccatum* reported by Gonzalez de Leon (1986). Because four recombinants were found among the 68 plants tested, the recombination frequency between the RAPD marker and the stunted growth-related gene was estimated to be about 6%.

Of the eight cultivars of *C. annuum* used in this experiment, six had the 610-bp fragment linked to the nuclear gene for stunted growth, the exceptions being 'Takanotsume' and 'Yatsubusa'. These first six belong to the family of sweet peppers; the latter to chili peppers. In future investigations, the 610-bp fragment will be used in the classification of cultivars in *C. annuum*.

Nuclear-cytoplasmic interaction has extensively been investigated for cytoplasmic male sterility in *Zea mays* and *Petunia* (Dewey et al. 1986; Young and Hanson 1987). These interactions are known to affect other developmental stages as well as pollen production (Jan 1992). Although we did not undertake molecular analysis of the cytoplasm, a number of reports have so far demonstrated that mitochondria as well as chloroplasts, are associated with plant vigor and growth. Newton et al. (1990) reported non-chromosomal stripe (NCS) abnormal growth mutants in maize. The mechanism is unknown by which the mitochondrial *cox2* gene was rearranged and their mRNA greatly reduced in the presence of nuclear genes in a inbred line. According to a report on another type of nuclear-cytoplasmic incompatibility (Newton and Courtney 1991), characteristic small kernels and low plant height was observed when the teosintes cytoplasms are present together with the homozygous recessive alleles of inbred maize. However, no difference in mitochondrial DNA, mRNA and synthesized proteins were detected in this case.

In our study, differences between the total proteins extracted from normal leaves and those from leaves of stunted plants in F_1s was not detected by two-dimensional electrophoresis (data not shown). As a next step, it will be interesting to analyze DNA, mRNA and the synthesized protein of their genes in mitochondria and chloroplasts.

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