Elimination of Somatic Chromosomes in Strawberry Plants by Treatment with *para*-Fluorophenylalanine

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Effects of *para*-fluorophenylalanine (PFP), an amino acid analogue, on callus induction and somatic chromosome reduction were investigated in strawberry plants ($2n=56$). High concentrations of PFP had an inhibitory effect on callus growth and RD_{50} was calculated to be 30mg/l for callus induction. Cytological study revealed that several plants which were regenerated from the calluses of several plant organs cultured on the medium containing PFP had the chromosome number of $2n=49$. The mechanism of this chemical agent underlying chromosome reduction was also discussed.

KEY WORDS: *Fragaria ananassa*, tissue culture, *para*-fluorophenylalanine, chromosome elimination.

Introduction

It has been known for many years that some chemical agents, for example colchicine, were able to double the chromosome number of a cell. This effect has been widely utilized in the field of plant breeding and new crop varieties have thus been successfully developed. The most advantageous aspect of the application of colchicine for genetic studies and plant breeding is that it can double the chromosome number of the plants readily compared with the methods previously used.

On the other hand chemical reduction of the chromosome number has been thought to be practically impossible to achieve. Although it had been known that plants with a reduced chromosome number could be induced among the regenerated plants through tissue culture or among the mutants treated with mutagenic agents on rare occasions, the occurrence of such phenomenon is difficult to predict and control. In order to develop the plants with a reduced number of chromosomes as monosomics, it is necessary to make hybrids and examine the chromosome number. Usually this set of investigations requires a long time and skill. Anther culture which is now extensively applied is one of the very effective methods to induce haploids. However the application of this technique to practical breeding is still limited to a few species and there are many problems to be overcome.

MORPURGO (in LHOAS 1961) demonstrated that haploids were frequently induced after treatment of diploid mycelia of *Aspergillus niger* with *para*-fluorophenylalanine (PFP). This discovery has been adopted by many researchers in genetic studies of microorganisms, such as *Aspergillus nidulans* (McCULLY and FORBES 1965), *Ustilago violacea* (DAY and JONES 1971) *Saccharomyces cerevisiae* (STRÖMNÆS 1967), etc., although haploids were not always obtained owing to species differences.

KNIGHT *et al.* (1963) noticed the occurrence of chromosome reduction in higher plants after PFP treatment. They observed aneuploid and haploid cells among root tip cells in vegetatively propagated *Ribes* hybrid. NITZSCHE (1973) observed even monoploid cells

when the amphidiploid between *Festuca pratensis* and *Lolium multiflorum* was treated with *meta*-fluorophenylalanine. NITZSCHE (1980) also found that some other halogenized amino acids had an effect similar to that of PFP. NIIZEKI (1976) obtained the strawberry plants with a reduced chromosome number following treatment with PFP which had been added to the tissue culture medium.

The effect of PFP on the somatic chromosome elimination in strawberry plants is reported in this paper.

Materials and Methods

Materials

The strawberry cultivar, "Hökōwase" (*Fragaria* × *ananassa* Duch. $2n=56$ $x=7$), was used throughout the study. Original materials were supplied by the Ornamental Experiment Station of Kanagawa Prefecture and were cultivated at the experimental field of the National Institute of Agricultural Sciences. Strawberry anthers were used to examine the effects of PFP on the callus induction and growth. For obtaining regenerated strawberry plants, calluses induced from various parts of the plants, such as shoot tips, petioles and anthers were used.

Culture methods

Anthers and shoot tips of strawberry plants were used without sterilization. In the case of shoot tips, young leaves were removed by sterilized pincettes and the centers of the tips were inoculated on the medium. Petioles of strawberry plants were immersed in a 1/300 of benzalkonium chloride solution for 30 seconds and then in 7% (w/v) of calcium hypochlorite solution for 5 minutes successively. After removing the epidermis, the petioles were cut into fragments of 3~4 mm long and placed on the medium. Linsmaier and Skoog's medium containing 0.8% of agar and 3% of sucrose and adjusted to pH 5.8 was used as basal medium. 0.2 mg/l of naphthaleneacetic acid (NAA) and 2.2 mg/l of 6-benzyladenine (BA) were added to the basal medium. PFP was added to each medium at the rate of 10 mg/l each time to reach a final concentration of 0, 5, 15, ~85 mg/l, respectively. There were approximately 50 anthers in each treatment and two or three anthers were inoculated together in every test tube. Callus characteristics such as diameter, color, etc. were examined after 40 days incubation. Shoot tips and petioles were also inoculated on the medium prepared as the same condition as the medium for anther culture. After two months incubation on the medium which contained PFP, inoculants were transferred to Linsmaier and Skoog's medium containing 2.2 mg/l of BA and without PFP. Regenerated plants were then transplanted to Linsmaier and Skoog's basal medium and were cultivated there for one month to obtain enough roots. Inoculants were incubated at 26~28°C with 12-hr-day length. Regenerated juvenile plants were carefully transplanted into nursery boxes and were grown there one month.

Microscopic examination

Root tips of strawberry plants were used for determination of the chromosome number. Collected root tips were immediately immersed in water and stored for 24 hours at 0°C. Thereafter root tips were fixed in Farmer's solution (ethanol 3: acetic acid 1) containing 0.5% of iron chloride. They were stained with acetocarmine solution and prepared by

squash method for microscopic examination.

Results

Dose effect of PFP on callus induction and growth

Plant materials used in this experiment were strawberry anthers. Only diploid somatic cells of anthers can divide and form calluses in the anther culture of strawberry at the condition used in this experiment. Therefore anther culture of strawberry supplies convenient and uniform materials for the study of somatic cells. Results of the effect of PFP on callus induction are shown in Fig. 1. In the control induction rate was approximately 90%. Among the treatments with low PFP concentrations such as 5 and 15 mg/l, the difference from the control could hardly be detected. Treatment with PFP at a concentration of 25mg/l, however, resulted in an apparent reduction of callus induction and no callus was induced at the concentration of 55mg/l. Thus an inhibitory effect of PFP on callus induction was clearly demonstrated and a half reduction dose (RD_{50}) of PFP was calculated to be 30mg/l. With the concentrations of PFP higher than 55mg/l a few calluses were induced. At the concentrations of 65, 75 and 85mg/l, the callus induction rate was 5.7, 10 and 10%, respectively. These calluses were induced from a part of each inoculant and seemed to be induced from a few cells survived by chance. Table 1 shows the effect of PFP on the callus growth. According to Table 1, growth of calluses was suppressed at the high concentrations of PFP. The

Fig. 1. Effect of PFP on the induction of callus from strawberry anthers.

Table 1. Effect of PFP on callus growth observed after 40 days of incubation

Concentration of PFP (mg/l)	Growth rate of the callus ¹⁾				Total
	±	+	++	+++	
0	6	13	12	6	37
5	4	8	19	8	39
15	8	15	10	5	38
25	3	11	6	3	23
35	5	3	2	0	10
45	5	0	0	0	5
55	0	0	0	0	0
65	2	0	0	0	2
75	2	1	1	0	4
85	2	1	1	0	4

1) Diameter of the callus (mm) ± : ~0.9, + : 1~1.9, ++ : 2~2.9
+++ : 3~

most of the calluses formed in applying PFP at the concentrations higher than 55 mg/l were about 2 mm and smaller in diameter and brownish or darkbrown in color as compared with the calluses formed in lower concentrations. Thus it was pointed out on the basis of the results of both Fig. 1 and Table 1 that the callus formation was almost the same among the control and the 5 and the 15 mg/l treatments and there was a threshold for callus formation at the concentration of 55 mg/l.

Chromosome number of the regenerated plants after treatment with PFP

Calluses which were induced from several organs such as anthers, petioles and shoot tips were used as materials for plant regeneration. As shown in Table 2, 57 of strawberry mature plants in total were obtained. Among the regenerated plants 19 were derived

Table 2. Induction of regenerated plants with a reduced chromosome number

Organs cultured	Concentration of PFP (mg/l)	Chromosome number				Total number of regenerated plants
		98	56	49	Others ¹⁾	
Shoot tip	0		14	3	2	19
	15	2				2
	75		3	10		13
Petiole	35			1		1
Anther	5		4	12	6	22
Total		2	21	26	8	57

1) Mixoploidies, etc.

from calluses of a shoot tip in the control, 22 from calluses of anthers with PFP at the concentration of 5 mg/l, 2 from calluses of a shoot tip at 15 mg/l of PFP, one from the callus of a petiole at 35 mg/l of PFP, and 13 from calluses of a shoot tip at 75 mg/l of PFP. Table 2 also shows the result of microscopic observation of the chromosome number in the regenerated plants. Among the 57 plants examined, 8 plants showed more than two of chromosome number or different number from the multiples of the basic number 7 among cells of each plant. Percentages of the regenerated plants with a chromosome number differing from that of the original strawberry plants were 17.7, 75.0, 100, 100 and 76.9% in the order of PFP concentration of 0, 5, 15, 35 and 75 mg/l, respectively. Thus it could be demonstrated that the induction of the plants with an aberrant chromosome number was far more frequent in the treatment of PFP than in the control. Most of the aberrant chromosome number showed $2n=49$, which corresponded to the chromosome number of the heptaploid strawberry plant. Two plants with a chromosome number of $2n=98$ were also observed at the 15 mg/l treatment. The origin of this type of plants could best be explained by the occasional doubling of the chromosome number of $2n=49$ in the course of tissue culture. Because one plant having the chromosome number of 49 and 98 was observed among the mixoploid plants. It is well known that doubling of the chromosome number sometimes occurs in the process of tissue culture. In contrast with the callus formation, the induction of plants with a reduced chromosome number seemed to be independent from the concentration of the agent applied.

Characteristics of the regenerated plants

It was rather difficult to distinguish the strawberry plants with a chromosome number of $2n=49$ morphologically from the original plants and the regenerated ones with the chromosome number of $2n=56$. They did not show any great difference on leaf shape, flowering time or plant type. Microscopic observation for stomata, however, revealed that the size of them were ranged in the sequence of $2n=98 > 2n=56 > 2n=49$. While the plants with the chromosome number of $2n=98$ differed conspicuously from the original variety or regenerated plants having the other chromosome numbers. They were smaller than the normal plant in leaves, petioles, *etc.* Leaves of them became rough and somewhat round in shape compared with the those of plants with the fewer chromosome number, whereas the flower became larger.

Discussion

On the basis of the results obtained in this experiment, the authors clearly demonstrated the effect of PFP on the reduction of the chromosome number in somatic cells and they succeeded in obtaining strawberry plants with a chromosome number of $2n=49$. NIIZEKI (1977) and NIIZEKI and FUKUI (1978) also reported the reduction of chromosome number using PFP in the wild rice, *Oryza punctata* subsp. *Schweinfurthiana* and *Oryza alta*. NITZSCHE (1980) was also able to obtain the plants with reduced chromosome number among the progenies of *Festuca-Lolium* hybrids treated with PFP. OMURA and AKIHAMA (1981) reported that they obtained a grape plant with reduced chromosome number. These results indicate that PFP can be applied as the agent of chromosome elimination to several species among higher plants.

Although the frequency of induction was low, plants with a reduced number of chromosomes were obtained in the control of this experiment. Cultivated strawberry plants ($2n=56$) are thought to be hybrids between *Fragaria chiloensis* ($2n=56$) and *F. virginiana* ($2n=56$). Both the octoploid parents are thought to be evolved from the original diploid species with the chromosome number of $2n=14$. It is thus conceivable that many genes were duplicated and consequently there must be a mechanism of gene compensation in the case of high polyploidy such as octoploidy. This might be the reason why there was no distinct difference between strawberry plants with the reduced chromosome number and the original ones. Such mechanism might also lead to natural fluctuation of the chromosome number under certain conditions such as tissue culture.

Regardless of the concentrations applied, PFP was frequently able to induce plants with reduced chromosome number. This phenomenon might be ascribed by the mechanism of PFP for chromosome reduction itself. Namely, PFP does not act directly upon chromosomes, but the malfunction of the 'division related proteins' which have been substituted by PFP for phenylalanine was thought to be the possible cause of chromosome elimination (SISKEN *et al.* 1972, SISKEN 1973). Therefore it might be conceivable that PFP dose would not a definitive factor on the effect of PFP on chromosome elimination but the other factors such as prolonged period of treatment might be more important. According to LHOAS (1968), the cytological mechanism of haploidization induced by PFP in *Aspergillus niger* can be explained on the basis of successive loss of chromosomes.

As the results, PFP could induce multiple monosomics and haploid nuclei among diploid nuclei. This observation in *Aspergillus niger* is in agreement to the results obtained in higher plants such as *Oryza* species (NIIZEKI 1977, NIIZEKI and FUKUI 1978), *Ribes* hybrid (KNIGHT *et al.* 1963), *Festuca-Lolium* hybrids (NITZSCHE 1980) and *Vitis* species (OMURA and AKIHAMA 1980, 1981). They observed not only haploids but also aneuploid cells or plants in their materials. It would thus be concluded that the gradual elimination of chromosomes at mitosis would eventually lead to the formation of cells with haploid chromosome number and/or a reduced chromosome number in higher plants. And under favorable conditions such as tissue culture or cutting back they could form plants with a reduced chromosome number.

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イチゴにおけるパラフルオロフェニルアラニン処理による体細胞染色体の減数

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アミノ酸の類似物質であるパラフルオロフェニルアラニン (PFP) が体細胞で染色体数を減少させることはコウジカビ, 酵母などでは知られているが高等植物ではわずかにスグリ, トールフェスクとイタリアンライグ

ラスの雑種などで知られていたにすぎない。著者らはイチゴの組織培養に PFP 処理を加味して PFP がカルス形成および体細胞染色体の減数に及ぼす効果について検討した。その結果、葯由来の 8 倍性カルスでは PFP の濃度の上昇に伴う著しい阻害効果がカルスの誘導率、生長率の両方に認められ、55mg/l の濃度でカルス誘導率は 0 になった。カルス誘導率を 50% にする濃度、 RD_{50} は 30mg/l と算定された。葯、葉柄、茎頂などの器官より誘導したカルスから 57 の再分化植物体を得た。PFP 処理区に由来する再分化個体には染色体数が 7 本減少した個体が多く含まれていることが明らかとなり、PFP がイチゴ体細胞における染色体減数に効果があると結論された。