Variation in the Chromosome Number of Cultured Shoot Tip of *Vitis* spp. by *p*-Fluorophenylalanine Treatment

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The effect of *p*-fluorophenylalanine (PFP) on the induction of variations in the chromosome number was investigated in cultured shoot tips of *Vitis* clones, including triploid and tetraploid cultivars and interspecific hybrids. A higher concentration (400 μ M) of PFP inhibited the growth of the shoot tips. The abnormal mitoses and the variations in the chromosome number including the induction of haploid and aneuploid cells were observed in the shoot tip cells treated with PFP. Variations in the chromosome number were also observed in the root tip cells of regenerated plants after PFP treatment. However, most of cells with abnormal chromosome number co-existed with normal cells. The average percentage of the abnormal cells was about 1.0% among total observed cells of all the clones examined. In most of the cases the abnormal chromosome number observed in the roots was euploids, regardless of the ploidy level of the clones examined. There were fewer aneuploids in root cells than in the shoot tip cells. In all the experiments, two variants (about 0.3% of the plants checked) had large sectors with reduced numbers of chromosomes in the shoots. One was a triploid-tetraploid variant from the tetraploid cultivar Kyoho, and the other was a diploid-triploid variant from the triploid clone KC-1.

KEY WORDS : *Vitis*, shoot tip culture, abnormal mitosis, chromosome reduction, *p*-fluorophenylalanine.

Introduction

Shoot tip culture has been studied and established as a technique for rapid propagation and for obtaining virus free materials in *Vitis* (BARLASS and SKENE 1978, SASAHARA *et al.*, 1981). The technique was also applied to obtain polyploids in grapes by the use of colchicine *in vitro*.

Tissue culture was also advantageously applied for the reduction of the chromosome number by the use of *p*-fluorophenylalanine (PFP) in *Fragaria x annanassa* and other crops (NIIZEKI and FUKUI 1983, FUKUI and NIIZEKI 1983). The effectiveness of *p*-fluorophenylalanine for the reduction in the chromosome number had been confirmed in grape after treatment of seeds and new shoots (OMURA and AKIHAMA 1980, 1981). It was suggested that the reduction of the chromosome number by PFP occurred through abnormal mitoses after the substantial incorporation of PFP into mitosis-related proteins (NODA 1982, OMURA *et al.* 1985). To improve the efficiency and clarify the characteristics of the reduction of chromosome number by PFP, we investigated the effect of PFP on the growth and variation in the chromosome number through shoot tip culture and compared the response among clones and ploidies in *Vitis*.

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Materials and Methods

A tetraploid ($2n=76$) grape cv. Kyoho (*Vitis labruscana*) was mainly used for the shoot tip culture in the experiments. Following cultivars and clones were also examined. Three diploid cultivars ($2x=38$): Delaware, Muscat of Alexandria ($2x$) and Perlette ($2x$). Two tetraploids ($4x=76$): Muscat of Alexandria ($4x$) and Perlette ($4x$). Three triploid clones ($3x=57$): KC-1 and KC-2, which were hybrids between Kyoho $\times$ Campbell Early, and KF-1, a variant obtained from Kyoho by PFP treatment (OMURA and AKIHAMA 1981). A mixoploid cultivar: Red Pearl ($2x+4x$). Allied species: *V. riparia*, *V. thunbergii*. A hybrid clone between Delaware $\times$ *V. thunbergii* (Dela. $\times$ *V. thunb.* -1) and a triploid hybrid between Kyoho $\times$ Couderc 3306 ($3x$).

Shoot tips which were aseptically excised to 0.2 to 0.3 mm in length were inoculated on initial liquid medium (pH 5.8), composed of Murashige and Skoog's basal medium supplemented with $5\text{ }\mu\text{M}$ of benzyladenine (BA). After two weeks of pre-culture at 28°C under a 16 hr light regime the shoot tips were transplanted to the medium which was the same as the initial liquid medium in the components but solidified with 0.7% agar. The growing cultured shoots were cut to 3 to 5 mm in length and subcultured in the agar medium in 40 to 50 day intervals. The basal part of the cultured shoot tips formed a shoot mass and calli with many short shoots during culturing. These shoot masses were subcultured as well as the subcultured shoot tips. At the time of subculturing, two or three elongated shoots in each culture were cut to about 1 cm in length and rooted in the rooting medium which consisted of half strength of MS basal medium supplemented with $0.1\text{ }\mu\text{M}$ naphthaleneacetic acid (NAA). The subculture of the shoot tips and rooting of elongated shoots were repeated until the fifth cycle of culture. The rooted plantlets were transplanted to vermiculite in pot and grown in a greenhouse for 30 to 40 days until the observation for chromosome number.

p-Fluorophenylalanine (DL-PFP, Sigma Chemical Co.) was added in the culture medium both of initial cultures and subcultures at the concentration of 0 (control), 25, 100 and $400\text{ }\mu\text{M}$ in the case of cv. Kyoho. For the other cultivars and clones, PFP was applied only in the concentration of $100\text{ }\mu\text{M}$. The initial growth of the shoot tips in the early stage of culture was estimated by determining the length of the shoots from the base to the top of the shoot tips at four and eight weeks after inoculation, using 24 to 26 tips for each concentration of PFP. For the observation of abnormal mitoses and chromosome numbers, 20 to 30 shoot tips were fixed with modified Carnoy's fluid after one month of culture and stained by aceto-orcein method. The chromosome numbers of regenerated plants were investigated in the root tips. If the abnormal plantlets were appeared, chromosome numbers were counted also in the shoot tip cells.

Results

(1) Effect of PFP on initial growth and mitosis of excised shoot tips of Kyoho

$400\text{ }\mu\text{M}$ of PFP severely inhibited the initial growth of the shoot tips of Kyoho, and only one tip could survive among the 24 examined (Table 1). On the other hand, 67,

Table 1. Effect of PFP on the initial growth of excised shoot tips of *Vitis labruscana* cv. Kyoho *in vitro*.

PFP concentration (μM)	Number of shoot tips		Shoot length at	
	Inoculated ¹⁾	Survived (%)	4 week (mm)	8 week (mm)
0	24	16 (67)	$2.9 \pm 0.8^2)$	7.7 ± 2.5
25	26	18 (69)	2.8 ± 0.8	4.4 ± 0.8
100	25	15 (60)	2.4 ± 1.1	6.7 ± 1.1
400	24	1 (4)	1.3	1.8

1) Shoot tips 0.3~0.5 mm were excised.

2) Mean $\pm$ standard errorTable 2. Effect of PFP on mitosis in cultured shoot tips of *V. labruscana* cv. Kyoho ($4 \times = 76$) after one month culture.

PFP concentration (μM)	Mitosis in shoot tips				Variation in chromosome number	
	Total observation		Mitotic index (%)	Abnormal mitosis ¹⁾ (%)	Frequency of cells ²⁾ (%)	Different number
0	30 tips	9985 cells	2.4	0.0	0.0	
25	30	9455	2.0	1.9	1.3	38, 45, 61
100	28	8904	1.7	3.6	1.5	28, 42, 152?
400	20	5960	1.4	1.7	1.1	57

1) Percentage of cells with C-mitosis, lagging chromosomes, multipolar mitosis, micronucleus and abnormal chromosome number to total metaphase-telophase cells in shoot tips.

2) Percentage of cells with abnormal chromosome number to total cells with normal chromosome number ($2n=76$).

69 and 60% of the shoot tips remained alive on the medium containing 0, 25 and 100 μM of PFP, respectively. Initial growth up to eight weeks after inoculation showed the same trend as the survival rate.

PFP treatment induced the abnormal mitoses in the shoot tip cells, including C-mitosis, lagging chromosomes, multipolar mitosis and micronucleus (Fig. 1 A). The frequencies of cells with abnormal mitoses were 1.9, 3.6 and 1.7% at 25, 100 and 400 μM , respectively (Table 2). The cells with abnormal chromosome numbers were also observed in shoot tips treated with PFP. The abnormal chromosome numbers ranged from 28 to 152(?), and dihaploid ($2x=38$) cells did not occur frequently in the cultured shoot tips treated with PFP. There were no abnormal mitoses in the 0 μM control.

(2) Effect of subculture and concentration of PFP on shoot tip culture in Kyoho

Two or three shoots from each ten tips were rooted at every subculture cycle, except for the 400 μM treatment due to the severe inhibition of shoot growth. Several root tip cells from regenerated plants had the different chromosome numbers from original tetraploid ($2n=76$) after the treatment with PFP at a concentration of 25 and 100 μM . In the 25 μM treatment, plant with abnormal chromosome number in root cells was obtained in each of the 1st, 2nd and 5th subculture of shoot tips. Total of four plants with abnormal chromosome number was formed through the 2nd, 3rd and 5th cycle of culture with 100 μM PFP. The frequencies of plants with an abnormal chromosome number in the root tips were 2.7% for 25 μM and 4.0% for 100 μM concentrations

Table 3. Variation of chromosome number in root tip cells of regenerated plants¹⁾ from subcultured shoots of cv. Kyoho on MS media containing PFP.

PFP concentration		Number of plants from cultured shoot tips at subculture					Total number of plants from subcultured shoot mass
		1 st	2nd	3 rd	4 th	5 th	
0 μ M	Total plants	23	19	25	23	20	35
	Variants	0	0	0	0	0	0
25	Total plants	23	28	19	21	21	38
	Variants ²⁾	1 (57)	1 (38)	0	0	1 (57)	2 (38, 47)
100	Total plants	18	21	21	21	19	29
	Variants	0	1 (38)	2 (38, 57)	0	1 (152 ?)	1 (19)
400	Total plants	2	4	2	5	3	2
	Variants	9	0	0	0	0	0

1) Chromosome number was counted in root tip cells of regenerated plants after potting

2) A few cells with variations in the chromosome number, which are shown in parentheses, co-existed with normal cells ($2n=76$)Table 4. Variation in the chromosome numbers in root tip cells of regenerated plants through first to third subculture of shoot tips on medium containing 100 μ M PFP

Clone (Chromosome number)	Variations in chromosome number observed	Ratio of variants (variant/total)	
		Plant ¹⁾	Cell ²⁾
Muscat of Alexandria (38)	19	1/14	2/146
Perlette (38)	76	1/27	1/325
Delaware (38)	—	0/35	0/387
KC-1 (57)	19, 38, 76	6/26	7/198
KC-2 (57)	—	0/19	0/169
KF-1 (57)	76	1/8	1/40
Muscat of Alexandria (76)	38, 57	3/18	3/203
Perlette (76)	38	2/30	3/358
Red Pearl (38+76)	—	0/30	0/176
<i>V. thunbergii</i> (38)	19, 76	5/35	7/389
<i>V. riparia</i> (38)	—	0/26	0/279
Dela. $\times$ <i>V. thunb.</i> -1 (38)	16, 19, 24, 32, 76	5/27	6/375
Kyoho $\times$ Couderc 3306 (57)	38	2/27	4/268

1) Variant plants which had at least 1 variant cell in the chromosome number in the root/total regenerated plants

2) Variant cells in the chromosome number/total cells counted.

(Table 3), but cells with abnormal chromosome number were not detected in the plants subjected to the 0 and 400 μ M treatment. By subculture of the shoot mass, callus was formed while shoot growth decreased. Abnormal chromosome numbers also occurred in root tip cells of regenerated plants in the frequency of 5.3% for the 25 μ M treatment and 3.4% for the 100 μ M treatment among the plants examined.

The variations in the chromosome numbers in the root cells included one monopleid, five diploid, three triploid, one octaploid and only one aneuploid ($2n=47$) cells. The abnormal cells were occurred in low frequency and co-existed with many normal cells in

the roots. However, a plant from the fifth cycle of shoot tip subculture which had been subjected to the 25 μ M PFP treatment, showed different leaf shape and growth habit. The chromosome number was 57 in some roots, but normal tetraploid as well as triploid cells were observed in the shoot tip cells.

(3) *Variation of chromosome numbers induced by PFP in shoot tip culture of Vitis clones*

The shoot tips of 14 cultivars and clones were cultured on 100 μ M PFP medium. Table 4 shows the results of the investigation in the chromosome numbers of the root tips in the regenerated plants through first to third subculture of shoot tips. Nine clones and hybrids out of the 14 examined exhibited variations in the chromosome numbers. Most of the variations occurred in a few cells which co-existed with normal cells in the root. These plants showed a normal appearance of the shoots and leaves. In all the clones examined, 34 cells with an abnormal chromosome number were detected among the 3390 cells counted. The relationship between the frequency of variated cells and the growth inhibition of shoots of clones was not clear. Also, there was no relationship between ploidy of clones and the frequency of variants. In diploid clones and hybrids, 16 abnormal cells were observed, accounting for 0.81% of the total. Haploid cells ($x=19$) were most frequent and tetraploid and aneuploid ones followed. In four triploids, twelve cells (1.8%) showed variations in eight diploids, followed by tetraploids and one monoploid. In the tetraploid clones, five diploid and one triploid cells were observed. In all the 34 cells showing variations in the chromo-

Fig.1. Abnormal mitosis and variant associated with PFP treatment through shoot tip culture in grape.

- A. Lagging chromosomes and micronucleus formation in shoot tip cells of Kyoho ($4x=76$).
- B. Chimerical variants derived from shoot tip culture of triploid clone KC-1 on the medium containing 100 μ M PFP.
- C. Mitotic figures in shoot tips of chimerical variants from KC-1 showing diploid and triploid cells.

some number, there were only three aneuploid cells, the others being euploid. Compared to most of the variant plants with a few cells showing variations, one variant from KC-1 exhibited a chimerical appearance in the shoot and leaf morphology and containing both diploid and triploid cells in the root and shoot (Figs. 1B and 1C).

Discussion

Through the tissue culture system, plants with a reduced number of chromosomes associated with PFP treatment were frequently obtained in *Fragaria x annanassa*, *Nicotiana tabacum* and other crops (FUKUI and NIIZEKI 1983). The effectiveness of PFP treatment in chromosome number was also confirmed in the shoot tip culture of *Vitis*, as 0.31% of the plants examined displayed chimeras in the chromosome number.

The reduced number of chromosome associated with PFP treatment has been reported in higher ploid plants, such as *Fragaria x annanassa* (8x, NIIZEKI and FUKUI 1983), *Oryza alata* (4x, FUKUI and NIIZEKI 1983), amphidiploid of *Ribes* hybrid (4x, knight *et al.*, 1963), *Festuca-Lolium* hybrid (4x, NITZSCHE 1980), *Triticum aestivum* (6x, FUKUI and NIIZEKI 1983), *Nicotiana tabacum* (4x, FUKUI and NIIZEKI 1983), *Ipomoea batatas* (6x, MURATA *et al.* 1983, 1984), except in the case of *Zea mays* (2x, BANKS *et al.* 1982). The present results in *Vitis*, in which large chimeras with a reduced chromosome number were derived from triploid and tetraploid but not from diploid forms, corresponded to the findings previously reported in other plants. The root cells treated with PFP at the early stage of germination of the seeds showed a wide range of variation in the chromosome number including many aneuploid cells in diploid cultivars (OMURA and AKIHAMA 1980, OMURA *et al.* 1985). The wide variation of the chromosome number induced by PFP was also observed in cultured shoot tip cells of Kyoho, while there were few aneuploid cells in the root tips of the regenerated plants. This observation suggests that several variant cells had been eliminated through the competition with normal cells during propagation and rooting process.

There were only two plants with a reduced number of chromosomes in the shoots by the PFP treatment of cultured shoot tips. However the results obtained indicate that the method for the reduction in the chromosome number through shoot tip culture of *Vitis* as well as *Ipomoea* (MURATA *et al.* 1983) may be a useful tool to manipulate the chromosome number. Further research is required to improve the efficiency of the method in order to obtain variants in the chromosome number in grape breeding through the comparison of the frequency of variants in the chromosome number.

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パラフルオロフェニルアラニン処理によるブドウの培養茎頂における染色体数の変異

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ブドウの培養茎頂に対して、パラフルオロフェニルアラニン (PFP) を処理し、染色体数の変異を調査した。4倍体品種巨峰 ($2n=76$) の茎頂を 0.2~0.3 mm の長さに切出し、PFP (0, 25, 100, 400 μ M) を含む MS 培地で培養した。初代培養では 400 μ M で生存率が低く、また、生育も劣った。また、25 μ M 及び 100 μ M では、対照区との間に生育の差が認められなかった (Table 1)。初代培養と同一濃度の PFP を含んだ培地で継代した茎頂で異常細胞分裂の発生を調査した。0 μ M 区では、異常分裂が全くみられなかったのに対し、25, 100 及び 400 μ M の各区とも、遅滞染色体、多極分裂、微小核などの発生が観察された (Table 2, Fig. 1 A)。また、処理区の茎頂では、染色体数が 28 から 152 までの広範囲に変異した細胞が観察された。

次に、巨峰の培養茎頂を PFP (0, 25, 100, 400 μ M) を含んだ MS 培地で継代培養し、伸長した枝を順次切り取って発根させ、再生個体の根端で染色体数を調査した。ここでは、継代部位について2種類の方法を用いた。伸長した枝の先端を3から5 mm に切り取って、継代を繰り返した場合、25 μ M 区では、第1, 2, 5回目の継代から、それぞれ1個体に染色体数の変異がみられた。また、100 μ M 区では、第2, 3, 5回目に、それぞれ1個体の変異が発生した。他方、伸長した枝を取り除いた苗条塊を継代した場合にも、25 及び 100 μ M 区で変異が観察された (Table 3)。しかし、いずれの方法でも、継代にともなって、変異の発生頻度が著しく向上するという傾向は認められなかった。また、これらの変異細胞のほとんどが、19, 38, 57, 152 の正

倍数性を示し、異数性は $2n=47$ の 1 細胞だけであった。これらの変異細胞のほとんどは、根端組織の正常細胞中に 1 から数細胞が混在するものであり、個体の外観には変異はみられなかった。しかし、 $25\mu\text{M}$ で第 5 回目の継代から発生した 1 個体は、茎頂部においても、3 倍性 ($2n=57$) に減数した細胞を約半数混在しており、 $3x$ と $4x$ とのキメラと考えられた。この個体は、外観上著しく変異していた。

$100\mu\text{M}$ の PFP を含む培地で、倍数性の異なる 14 の品種、系統、近縁種及び雑種の茎頂を培養し、再生個体における染色体数の変異を比較した。このうち、9 系統で根端細胞に変異が観察されたが、倍数性による発生頻度の差異は明らかでなかった。全供試系統を通じて、34 の変異細胞（観察細胞数の 1.0%）がみられたが、異数性の 3 細胞を除いては、正倍数性を示した。変異した染色体数では、2 倍体からは半数性 ($x=19$)、3 倍体及び 4 倍体では 2 倍性 ($2x=38$) の頻度が高かった (Table 4)。また、これらの変異細胞も、根端中に 1 から数細胞が正常細胞中に混在して発生するものであったが、3 倍体系統 KC-1 からの 1 変異個体は、地上部においても $2x$ と $3x$ とのキメラを示した (Figs. 1B, C)。

以上の実験を通じて、ブドウの培養茎頂への PFP 処理により、2 個体（調査数の 0.31%）の安定した染色体減数性キメラがえられ、PFP を茎頂培養系における染色体操作手法として利用できることが確認された。しかしながら、処理中の茎頂では多様な染色体数の変異が観察されるのに対し、再生個体では異数性の頻度が低下することは、変異細胞の多くが増殖及び発根過程で消失することによると推察された。