**Artificial induction of clonal tetraploids in an
annual plant, *Haplopappus gracilis* ($2n=4$),
by using tissue cultured shoot primordia¹⁾**

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ABSTRACT

Artificial clonal tetraploids were induced with colcemid using tissue cultured shoot primordia in an annual plant, *Haplopappus gracilis* ($2n=4$). The method for the successful induction of tetraploid in tissue cultured shoot primordia was treatment with 0.5 mg/ml of colcemid in liquid medium for 3 days. During the treatment the frequency of the tetraploid cells in shoot primordia increased gradually. On 30 days after the treatment, the majority (about 85%) of the metaphase cells in each shoot primordium were tetraploid. The shoot primordia on 30 days after the treatment were regenerated into plants, of which the almost all were tetraploid. Tetraploid plants obtained by this method seem to be one clone. It is concluded that direct induction of clonal tetraploids is possible by using the shoot primordia.

1. INTRODUCTION

In recent years artificial polyploids have been induced by using plant tissue cultures, such as callus from cell suspension (Heinz and Mee 1970), somatic embryo (Loh and Ingram 1983), meristem tissue (Sangthai *et al.* 1973) and the others, which were treated with colchicine. In the case of callus, the cells are genetically unstable and many variations arise (Sunderland 1973). Consequently, it is difficult to induce the polyploid for the purpose and it is also difficult to regenerate the cultures of many kinds of plants into plantlets (Halperin 1986). On the other hand, protocorm-like body (PLB) or shoot multiples by meristem culture is genetically stable and it is possible to regenerate them into plantlets. This method is available to induce polyploids of *Dendrobium* (Sangthai *et al.* 1973), and other orchids belonging to the perennial plants, but the induction of polyploids from PLB in useful annual plants has not yet been successful. One of

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the present authors reported a new micro-propagating mass of cells, called "shoot primordium", in the annual *Haplopappus gracilis* ($2n=4$) (Tanaka and Ikeda 1983). Tissue cultured shoot primordia derived by shoot tip cloning have proliferated rapidly maintaining chromosomal and genetical stability for many years. Shoot primordia also have the ability to easily regenerate into plantlets. The shoot primordium derived from a shoot tip is globular or dome-shaped, and appears to be turning into an adventitious shoot. It forms a mass of shoot primordium, and grows rapidly in size and number. Shoot primordia can be maintained and proliferated in this form for many years, and it is possible to clonally proliferate many kinds of annual plants perennially by using the shoot primordia. These shoot primordia are also genetically stable. Therefore it is possible to vegetatively preserve the chromosome-types and genotypes such as aneuploid, triploid and interspecific F_1 -hybrid of annual plants (Tanaka 1983, Tanaka *et al.* 1983), although they are difficult to possess their genetical stability when using other vegetative cultures. In the present paper the results of the experiment on the induction of artificial tetraploids by using shoot primordia will be reported.

2. MATERIAL AND METHODS

(1) *Subculture and colcemid treatment on shoot primordia of Haplopappus gracilis* ($2n=4$)

For the material, the tissue cultured shoot primordia initiated by Tanaka and Ikeda (1983) were used. The shoot primordia were originated from a shoot-tip explant of *Haplopappus gracilis* (Nutt.) Gray ($2n=4$), and they have been maintained by subculturing every two to four weeks for 52 months since January, 1981. The shoot primordia were maintained in the modified liquid medium of Murashige-Skoog (Murashige and Skoog 1962) supplemented with 2.0 mg/l 6-benzylaminopurine (BAP) and 30 g/l sucrose (MS medium), at pH 5.7 before autoclaving. Then, they were incubated in a test tube (30 mm in diameter) at 22°C under 2,000 to 10,000 lux illuminated continuously by halogen lamp, on a gyrated drum rotating at 2 cycles/min (cf. Tanaka and Ikeda 1983). The shoot primordia subcultured were incubated in the modified MS medium supplemented with 0.0001 to 1 mg/ml of colcemid (colcemid medium) at the same condition as the previous for 0 to 10 days. After the treatment, the shoot primordia were washed twice with fresh liquid MS medium without colcemid and replaced in another fresh liquid MS medium subculturing every two weeks thereafter for recovery and regeneration.

(2) *Regeneration of plantlets from shoot primordia*

The shoot primordia which were subcultured after colcemid treatment were regenerated into plantlets. A few shoot primordia were separated from a mass of

shoot primordia and each piece of shoot primordia was placed on basal medium of Gamborg's B5 (Gamborg *et al.* 1968), supplemented with 0.2 mg/l BAP, 20 g/l sucrose and 9 g/l agar, at pH 5.7. The condition was subjected to stationary culture at 22°C under the illumination of 4,000 lux fluorescence tubes for 16 hrs and in the dark for 8 hrs per day.

(3) *Cytological examination*

The frequency of mitotic cells and the chromosome numbers were observed in the shoot primordia and regenerated shoots. In order to count the frequency of mitotic cells, shoot primordia were fixed in modified Carnoy's solution (ethanol: glacial acetic acid: chloroform=2:1:1) for 3 to 24 hrs at 4°C. Fixed materials were double-stained in Schiff's reagent (Feulgen method) and in 2% aceto-orcein and squashed. In order to count the number of chromosomes, shoot primordia and shoots were pretreated with 0.002 M 8-hydroxyquinoline at 18°C before fixation. In shoot primordia, pretreatment was made for 3 hrs, and in shoots, for 1.5 hrs. The method of fixation and staining was the same as that used in the observation of frequency of mitotic cells. The frequency of mitotic cells and polyploid cells were calculated by respectively counting minimum of 3000 mitotic cells and 100 metaphase cells.

3. RESULTS

(1) *The optimum concentration of colcemid treatment to inhibit nuclear division*

In order to determine the optimum concentration of colcemid in liquid medium for the induction of tissue cultured tetraploid shoot primordia, the frequency of mitotic cells, especially of metaphase, anaphase and telophase, were counted at each of the five concentrations from 0.0001 to 1 mg/ml of colcemid during successive 10 treatment days (Fig. 1).

On the medium without colcemid (control), both frequencies of metaphase cells and anaphase+telophase cells remained fairly constant at 0.3 to 0.8% throughout the treatment days (Fig. 1A). By treating with 0.0001 mg/ml of colcemid, the lowest concentration of colcemid medium, the frequency of metaphase cells had been 0.6 to 0.7% until day 2 which was almost similar to that of the control (Fig. 1B). But after day 3, it slightly decreased, and at day 6 to 0.2%, and continued to be low. The frequency of anaphase+telophase cells with this concentration of colcemid was 1.1% at day 1, decreasing to 0.4% at day 4, and then later slightly increased to about the same frequency as the control (Fig. 1B). With 0.001 mg/ml of colcemid, the frequency of metaphase cells showed a slight increase, thus, quite similar to that with 0.0001 mg/ml treatment (Fig. 1C). With 0.01 mg/ml of colcemid, the frequency of metaphase cells and anaphase+telophase were similar up to day 5, somewhat higher than that of the control. Later both frequencies decreased (Fig. 1D). With a slightly higher concentration, 0.1 mg/ml

Fig. 1. Changes in the percentage of mitotic cells during the colcemid treatment in tissue cultured shoot primordia of *Haplopappus gracilis* ($2n=4$).

of colcemid, a maximum frequency of 1.2% was obtained at day 1, which was 3 times as that in the control. Later it markedly decreased almost to 0. But, the frequencies of anaphase+telophase cells showed no increase and gradually decreased to 0 (Fig. 1E). On the highest concentration of colcemid medium, 1 mg/ml, the frequency of metaphase cells indicated the highest value of 2.8% among all the treatments at day 1, which was 7 times as that in the control, but it markedly decreased almost to 0 at day 10. However, few anaphase and telophase

cells were observed with 1 mg/ml of colcemid (Fig. 1F).

From the result shown in Fig. 1, it was found that the treatment with 0.1 to 1 mg/ml of colcemid effectively inhibited nuclear division between metaphase and anaphase. The treatment with 0.1, 0.25, 0.5, 0.75 and 1 mg/ml of colcemid was carried out, in order to determine the optimum concentration of colcemid for the induction of tetraploid shoot primordia.

(2) *Treatment time with colcemid and the chromosome number of the cells in shoot primordia*

The chromosome number of all metaphase cells were counted in the shoot primordia treated with 0.1 to 1 mg/ml of colcemid. Each frequency of diploid ($2n=4$), tetraploid ($2n=8$), octoploid ($2n=16$) and hexadecaploid cells ($2n=32$) were counted during successive 10 treatment days. The results were as follows (Figs. 2 and 3): On a control, 100% were diploid cells during 10 days (Fig. 3A). With 0.1 mg/ml of colcemid, few tetraploid cells and octoploid cells were observed. The frequency of tetraploid cells ranged from 1.7 to 9.6% after day 1, that of octoploid cells ranged from 1.0 to 1.9% after day 4 (Fig. 3B). With 0.25 mg/ml of colcemid, the frequency of diploid cells gradually decreased during treatment days and the value was 8.9% at day 8. The frequency of tetraploid cells increased gradually and the highest value was 63.6% at day 3 and then it decreased to 35.5% at day 8. Octoploid cells appeared from day 2, the frequency increased gradually and reached to 19.4% at day 8 (Fig. 3C). With 0.5 mg/ml of colcemid, it showed

Fig. 2. Mitotic metaphase chromosomes in the cells of tissue cultured shoot primordia treated with colcemid, in *Haplopappus gracilis* ($2n=4$). A, diploid cell ($2n=4$). B, tetraploid cell ($2n=8$). C, octoploid cell ($2n=16$). D, hexadecaploid cell ($2n=32$). Bar indicates 10 μ m.

Fig. 3. Changes in the percentage of polyploid cells during the colcemid treatment in tissue cultured shoot primordia of *Haplopappus gracilis* ($2n=4$).

the same tendency as 0.25 mg/ml, but the frequency of tetraploid cells was a little higher and was 47.9% at day 6 (Fig. 3D). Similar tendency was found in 0.75 mg/ml of colcemid, but no diploid cell was observed at day 8 (Fig. 3E). 1 mg/ml

of colcemid had also similar tendency as 0.25 to 0.75 mg/ml, diploid cells markedly decreased after day 3 (Fig. 3F). At the last day (day 10), no mitotic cell was observed in 0.25 to 1 mg/ml of colcemid. This result suggests no significant difference in polyploidization among the concentrations of 0.25 to 1 mg/ml of colcemid, indicating that the treatment with 0.25 to 1 mg/ml of colcemid is optimum to induce polyploid shoot primordia. Fig. 3 also suggests that shoot primordia of various ploidy levels may be induced depending on the duration of treatment. The maximum frequency (44.7 to 62.0) of tetraploid cells was observed on day 3 in 0.25 to 1 mg/ml of colcemid medium during the treatment. These results indicate that the treatment with 0.25 to 1 mg/ml of colcemid for about 3 days is optimum for the induction of tetraploid shoot primordia of *Haplopappus gracilis*.

(3) Induction of tetraploid shoot primordia and their cloning

Based on the results of the preceding experiment, two conditions of colcemid treatment, 0.5 mg/ml of colcemid for 3 days and 1 mg/ml for 2 days were selected to induce tetraploid shoot primordia. Shoot primordia were recovered in fresh liquid MS medium without colcemid after the treatment. Number of metaphase chromosomes were counted in the cells of shoot primordia during and after the treatment, and the frequency of tetraploid cells in each shoot primordium were examined (Tables 1 and 2). In Tables 1 and 2, the actual numbers of the cells in each ploidy level found in each shoot primordium are shown. A wide variation can be seen in the frequency distribution among the shoot primordia after the colcemid treatment.

During the treatment, all shoot primordia were mixoploid, and after that the more shoot primordium proliferated, the more the frequency of tetraploid cells increased (Tables 1 and 2). Recovery from the two conditions of colcemid treatment showed different tendencies: On 1 and 3 days after the treatment with 0.5 mg/ml of colcemid for 3 days, all shoot primordia were mixoploid, thereafter the frequency of tetraploid cells increased (Table 1). Among the total of nine shoot primordia, on 30 days after the treatment, five shoot primordia were tetraploid, three were mixoploid and the other was diploid. Among the total of ten shoot primordia, on 60 days after the treatment, seven shoot primordia were tetraploid. Among the total of ten shoot primordia, on 120 days after the treatment, six shoot primordia were tetraploid. In contrast to the above condition, by the treatment with 1 mg/ml of colcemid for 2 days, all of the five shoot primordia, were mixoploid on 1 day after the treatment (Table 2). Among the total of seven shoot primordia, on 30 days after the treatment, only one shoot primordium was tetraploid, five were mixoploid and the other was diploid. Among the total of nine shoot primordia, on 60 days after the treatment, only two were tetraploid.

According to this result, by the treatment with 0.5 mg/ml of colcemid for 3 days, all shoot primordia were mixoploid during the treatment. But after the

Table 1. *The number of ploidy cells in tissue cultured shoot primordia of Haplopappus gracilis (2n=4) during and after the treatment with 0.5 mg/ml of colcemid for 3 days*

Treatment time (days)	Recovery time (days)	Shoot primordium no.	No. of cells with following chromosome number, 2n (ploidy)					total	% of tetraploid cells
			4 (2x)	8 (4x)	16 (8x)	32 (16x)	others		
0		1	1					1	0
		2	2					2	0
		3	2					2	0
		4	4					4	0
		5	5					5	0
		6	6					6	0
		7	16					16	0
1.5		1	23					23	0
		2	39	4				43	9
		3	19	4				23	17
		4	8	2				10	20
		5	21	8				29	28
3	0	1	7	5	1			13	15
		2	9	11				20	55
		3	6	10	5			21	48
		4	10	15	2			23	65
		5	3	10	3			16	63
		6	3	9	5			17	53
	1	1	12	9				21	43
		2	5	13	5			23	56
		3	10	32	6			48	67
		4	3	16	6	1		26	62
		5	3	22	3	1		29	76
	3	1	2		2			4	0
		2	1	5	1			7	71
		3		9	1	1		11	82
		4		7	4			11	64
		5		4	3			7	57
	30	1	5					5	0
		2		6				6	100
		3		12				12	100
		4		9				9	100
		5		2				2	100
		6		17				17	100
		7		21	1			22	96
		8		6	1			7	86
		9		5	1			6	83

Table 1. (continued)

Treatment time (days)	Recovery time (days)	Shoot primordium no.	No. of cells with following chromosome number, 2n (ploidy)					% of tetraploid cells	
			4 (2x)	8 (4x)	16 (8x)	32 (16x)	others		total
	60	1	4					4	0
		2	1	1				2	50
		3		5				5	100
		4		5				5	100
		5		7				7	100
		6		13				13	100
		7		14				14	100
		8		18				18	100
		9		21				21	100
		10				4		4	0
	120	1	4				1*	5	0
		2	1	11				12	92
		3		3				3	100
		4		6				6	100
		5		8				8	100
		6		14				14	100
		7		14				14	100
		8		25				25	100
		9		22			1**	23	96
		10					2***	2	0

* 2n=7, ** 2n=9, *** 2n=7.

Table 2. The number of ploidy cells in tissue cultured shoot primordia of *Haplopappus gracilis* (2n=4) during and after the treatment with 1 mg/ml of colcemid for 2 days

Treatment time (days)	Recovery time (days)	Shoot primordium no.	No. of cells with following chromosome number, 2n (ploidy)					% of tetraploid cells
			4 (2x)	8 (4x)	16 (8x)	others	total	
0		1	1				1	0
		2	2				2	0
		3	2				2	0
		4	4				4	0
		5	5				5	0
		6	6				6	0
		7	16				16	0
1		1	7				7	0
		2	10				10	0
		3	12				12	0

Table 2. (continued)

Treatment time (days)	Recovery time (days)	Shoot primordium no.	No. of cells with following chromosome number, 2n (ploidy)					% of tetraploid cells
			4 (2x)	8 (4x)	16 (8x)	others	total	
		4	15				15	0
		5	32				32	0
		6	5	1			6	17
2	0	1	20	4	1		25	16
		2	6	2			8	25
		3	8	3			11	27
		4	18	8			26	31
		5	13	6			19	32
		6	6	3			9	33
		7	7	4			11	36
	1	1	5	3			8	38
		2	6	7	1		14	50
		3	5	7	3		15	47
		4	19	36	10		65	55
		5	5	17	1		23	74
	3	1	10	11	3		24	46
		2	6	10	3		19	53
		3	2	7			9	78
		4	5	15	4		24	63
		5	3	11	2		16	69
		6	1	7	1		9	78
	30	1	7				7	0
		2	9	1			10	10
		3	10	4			14	29
		4	9	5			14	36
		5	3	20			23	87
		6	1	10			11	91
		7		7			7	100
	60	1	8				8	0
		2	12				12	0
		3	14	2			16	13
		4	2	2			4	50
		5	1	18			19	95
		6		11		2*	13	85
		7		2	1	1**	4	50
		8		4			4	100
		9		4			4	100

* 2n=6, 2n=7, ** 2n=7.

treatment the frequencies of tetraploid cells were gradually increasing in each shoot primordium after the treatment, and since 30 days after the treatment, the majority (about 80%) of the metaphase cells in each shoot primordium had been tetraploid cells (Fig. 4B). It is suggested that a new shoot primordium developed independently of each other, from few cells in the outermost layer of an old shoot

Fig. 4. Tissue cultured shoot primordia and the regenerated plants of *Haplopappus gracilis* ($2n=4$). A, cultures of the masses of shoot primordia which have been subcultured for 6 years since the original induction (Tanaka and Ikeda 1983). B, the shoot primordia 60 days after the treatment with 0.5 mg/ml of colcemid for 3 days. Majority of their cells are tetraploid. C, diploid plants regenerated from A. D, tetraploid plants regenerated from B. The tetraploids grew more rapidly than the diploids in C. Bars indicate 1 cm.

primordium (Cf. Tanaka and Ikeda 1983). In contrast, by the treatment with 1 mg/ml of colcemid for 2 days, the frequencies of tetraploid cells showed little increase (about 50%) in each shoot primordium after the treatment and remained mixoploidy.

(4) *Regenerated shoot and its chromosome number*

Shoot primordia treated with 0.5 mg/ml of colcemid for 3 days and recovered for 0, 30, 60 and 120 days after the treatment were placed on regeneration medium. Chromosomes of shoot tips of the young regenerated shoots were counted (Fig. 4C, D, Fig. 5, Table 3).

Fig. 5. Mitotic metaphase chromosomes in the shoot tip cells of the four tetraploid plants regenerated from four clonal tetraploid shoot primordia of *Haplopappus gracilis*. A, B, C, and D, tetraploid showing $2n=8$. Bar indicates $10\ \mu\text{m}$.

Table 3. *The polyploid clones regenerated from tissue cultured shoot primordia of Haplopappus gracilis ($2n=4$) after the treatment with 0.5 mg/ml of colcemid for 3 days*

Concentration of colcemid (mg/ml)	Recovery time of shoot primordia (days)	No. of plants with following chromosome number, $2n$ (ploidy)				Percent of tetraploid
		4 (2x)	6 (3x)	8 (4x)	mixoploid	
(control)	0	10				0
	30	7				0
	60	8		1		11
	120	9			1	0
0.5	0	1	1	5	3	50
	30			8		100
	60	1		8	1	80
	120			9		100

Chromosomes of 10 shoots out of the adventitious shoots regenerated from the shoot primordia directly after the treatment, were counted. Among them, five were tetraploid, three were mixoploid, one was diploid and the other was

hexaploid, 50% being tetraploid. In contrast, eight shoots regenerated from the shoot primordia recovered for 30 days after the treatment were all tetraploid. Among the total of ten shoots, regenerated from the shoot primordia recovered for 60 days after the treatment, eight (80%) were tetraploid. Nine shoots, regenerated from the shoot primordia recovered for 120 days after the treatment, were all tetraploids.

From this result, it was found that the shoot primordia recovered for more than 30 days should be used in order to effectively obtain tetraploid plantlets. Tetraploid plants obtained by this method are regarded as one clone. Therefore, it is concluded that direct induction of clonal tetraploids is possible by using tissue cultured shoot primordia.

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