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I. Fujishige · R. Tanaka · K. Taniguchi

Efficient isolation of non-chimeric tetraploids artificially induced in a stable culture of *Haplopappus gracilis*

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Abstract A method for reducing cytochimerism and inducing homogeneous tetraploids in *Haplopappus gracilis* (2n = 4) was developed in which masses of shoot primordia treated with 0.5 mg/ml of colcemid for 3 days were cut into small meristematic domes. All of the shoot primordia sampled just after the colcemid treatment were cytochimeras that were mixoploids of 2x, 4x and 8x cells. However, when they were allowed to recover in a colcemid-free medium, the frequency of 4x cells spontaneously increased in most of the shoot primordia. Thirty days after the recovery, chimeric masses containing shoot primordia, each of which consisted uniformly of 4x or 2x cells, were observed. In order to obtain a completely homogeneous tetraploid mass, we then cut these primordia into small pieces, each of which had approximately one meristematic dome. Subsequent to this homogeneous tetraploid masses were easily obtained. Tetraploid shoot primordia could propagate with chromosomal stability over a year, and plants regenerated from these tetraploid shoot primordia were also completely tetraploid. These results show that non-chrimeric masses can be easily isolated from artificially induced cytochimeras using masses of shoot primordia as material.

Key words Plant tissue culture · *Haplopappus gracilis* · Artifical tetraploid · Chimera · Genetic stability

Introduction

A chimera, which may be defined as two or more groups of cells that are genetically different with respect to ploidy, is often found in plants. Sometimes its presence is more valuable than that of the normal type, especially in an ornamental plant (Pierik and Steegmans 1983), but it is also an unwelcome result when uniform traits are needed. In the case of cultured tissues, chimeras are often induced spontaneously by somaclonal variations or artificially by mutagen treatment that lead to "cytochimera", and this can be disadvantageous for genetically stable propagation in vitro, except in the special case as a means of breeding.

It is generally said that various factors affect the genetic stability of cultured tissues throughout the subculture, the most important being the starting material, the physical and chemical conditions for the culture and the aging of the culture (Heinz and Mee 1971; Sacristán 1971; Singh and Harvey 1975; Nandi et al. 1977; Bayliss 1980; Fukui 1983; Ashmore and Shapcott 1989). Tanaka and Ikeda (1983) avoided a spontaneous induction of the somaclonal variations and reported "shoot primordia" as being one of the most genetically stable tissues. Masses of shoot primordia are successively multiplicating meristematic domes induced from a shoot tip. Although there have been many discussions as to whether the name "shoot primordia" is appropriate or not, cultured tissues were certainly found that had high regenerative potential, and the chromosomal stability was subsequently studied in detail. We consider that shoot primordia masses have characteristics similar to those of "enlarged apical domes" (Wakizuka and Yamaguchi 1987) and "meristematic nodules" (Aitken-Christie et al. 1988). Some of the commom features among these tissues are the multiple dome- or nodular-shaped morphology, capacity for long-term mass propagation, high regenerative potential and genetic stability.

We have studied the cytogenetical properties of masses of shoot primordia. It has been found that the masses can propagate stably for at least 84 months, maintaining the original chromosome number, without losing any regenerative potential (Tanaka et al. 1988a). Shoot primordia masses were also induced from suspended cells originating from callus, not only from shoot

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I. Fujishige · R. Tanaka · K. Taniguchi (✉)
Laboratory of Plant Chromosome and Gene Stock, Faculty of Science, Hiroshima University, Higashi-Hiroshima, 739 Japan

tips, and some of these homogeneously contained unusual karyotype mutations (Taniguchi and Tanaka 1989; Taniguchi and Fujishige, in preparation). Artificial tetraploid shoot primordia were directly induced from diploid shoot primordia masses by a colcemid treatment, and tetraploid plantlets were regenerated from the tetraploid shoot primordia (Tanaka et al. 1988b). Although chromosome doubling by colchicine or colcemid itself is a classical breeding technique, it is also useful as a model system by which to study cytological chimerism. The present paper reports on an efficient method for reducing cytochimerism and isolating homogeneous tetraploids using a stable propagation of shoot primordia. The process of reducing the chimerism from the mixoploid state is cytogenetically studied here.

Materials and methods

Materials

Masses of shoot primordia of *Haplopappus gracilis* (Nutt.) Gray (Strain KH-1) were used as material (Fig. 1A, B). They were initiated from a shoot tip which had a shoot apical meristem and the two youngest leaf primordia. The shoot primordia masses could be multiplicated without undertaking further differentiation. The propagation process of the shoot primordia masses was as follows: (1) small regions of preferential cell division initiated and grew into primary shoot primordia; (2) the primary shoot primordia developed into secondary shoot primordia; (3) new primary shoot primordia occurred de novo on the surface of the secondary shoot primordia.

Culture

Masses of shoot primordia were initiated and subcultured in 25 ml of a Murashige-Skoog liquid medium (Murashige and Skoog 1962) supplemented with 2.0 mg/l of 6-benzylaminopurine (BAP) and 30 g/l sucrose, pH 5.7–5.8. These were incubated in a test tube 30 mm in diameter, on a gyrated drum rotating at 2 cycles/min, at 22 °C under 10,000 lux of continuous illumination by a halogen lamp. Each mass propagated approximately twofold in total volume in 2 weeks and separated into a few masses spontaneously or upon minimal dissection with a spatula. These masses had been maintained by subculturing every 2 weeks for 5 years, keeping the original chromosome number $2n = 4$ until the present experiment. They also possessed regenerative potential, although at the time of this investigation it took a longer time to be regenerated into whole plants than it did in the early days of the culture.

Colcemid treatment and recovery culture

The tissues were subcultured 2 days before colcemid treatment in order to activate cell divisions. Colcemid (Sigma) was added to the medium at a final concentration of 0.5 mg/ml, and the solution was sterilized by filtering. The masses of shoot primordia were incubated in the colcemid medium for 3 days under the same conditions as the subculture. After the treatment, the masses were rinsed 3 times with a fresh medium and then subcultured again, this time in a colcemid-free medium for recovery.

Cutting masses of shoot primordia

The masses of shoot primordia were axenically cut into small pieces (approximately 1 mm in diameter) with tweezers on a dish. They were then cultured together in 10 ml of medium in a test tube.

Regeneration

Regenerated plants from the shoot primordia were obtained on a solid 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 22 °C under an illumination of 4,000-lux fluorescence tubes for 16 h a day.

Observation of metaphase cells

Chromosome numbers were counted in the metaphase cells of the shoot primordia and regenerated shoots. The whole mass of shoot primordia and shoot tips of the regenerated plantlets were first stained by Feulgen's method. The domes that were approximately 0.5–1 mm in diameter and stained pale pink were the secondary shoot primordia, and the small dark-stained domes or regions on their surface were the primary shoot primordia that were undergoing active mitosis (Fig. 2). In order to observe chromosome numbers, the primary shoot primordia were dissected out with tweezers under a binocular. One tissue segment was prepared on a glass-slide by the squash method after an additional staining with 1% aceto-orcein for 15–20 min.

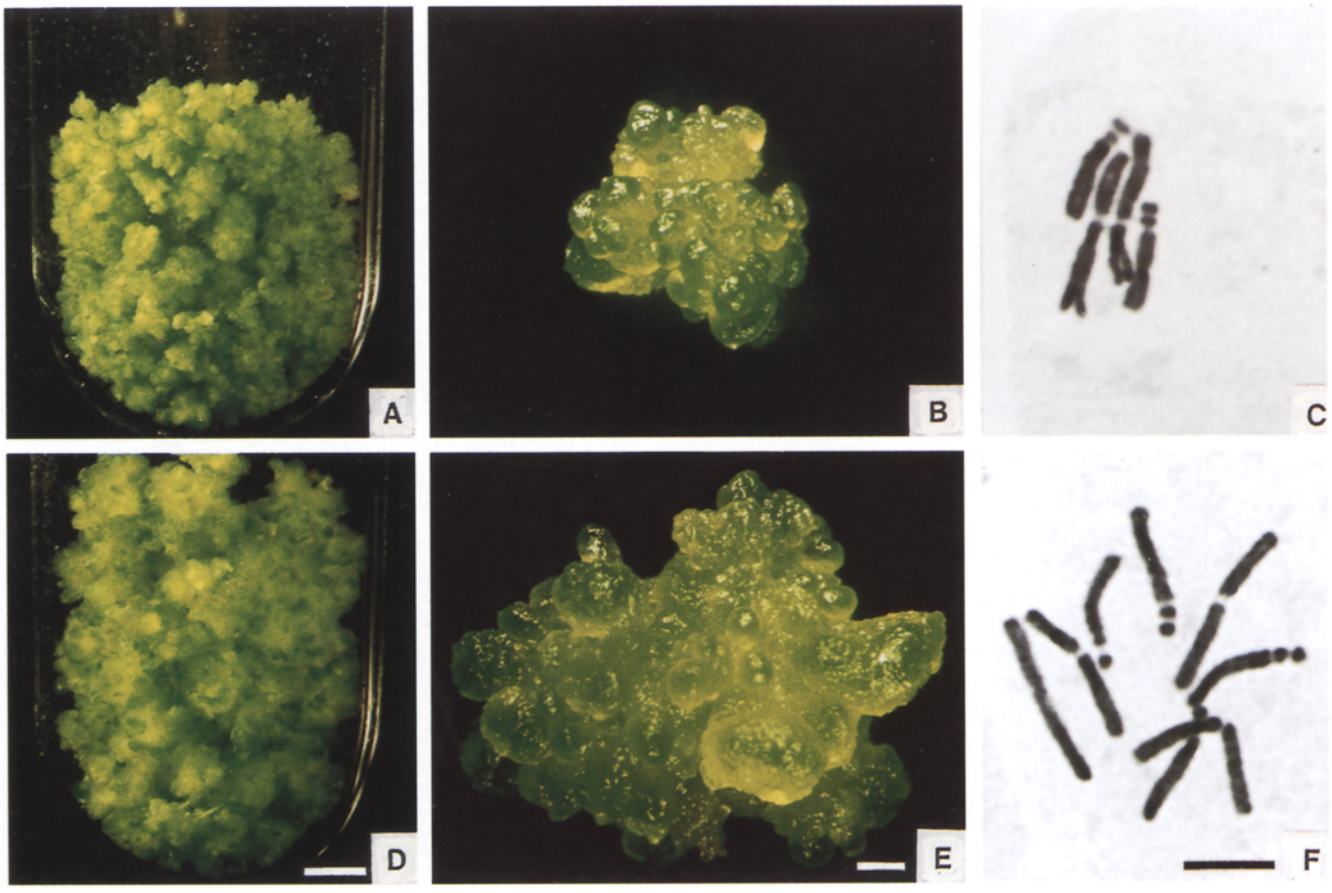
Criteria for judging the ploidy level of each shoot primordium

In each shoot primordium the number of metaphase cells observed for chromosome number was limited to a maximum of 20–30 cells. Therefore, we did not consider it valid to conclude that a shoot primordium was mixoploid when, for example, there were one diploid cell and 29 tetraploid cells in that primordium. The shoot primordia of *H. gracilis* normally show a stability of 98–100% diploid cells when a sufficient number of cells are counted (minimum of 100 metaphase cells). Therefore, as a matter of convenience, the ploidy level of the artificially treated shoot primordium was judged on the basis of the following criteria: a shoot primordium consisting of diploid cells or tetraploid cells at a frequency above 90% was considered to be a uniform diploid or tetraploid shoot primordium, respectively; and below 90%, as a mixoploid one. Furthermore, the mass was regarded as a homogeneous diploid or tetraploid only when all of the six shoot primordia observed were diploid or tetraploid, respectively, and as a chimeric mass for the others.

Results

Figure 3 shows the effects of the colcemid treatment with or without recovery period on the ploidy level of the shoot primordia of *H. gracilis*. The shoot primordia were subjected to a chromosome count on days 1.5 and 3 of the colcemid treatment and days 1.5, 3, 6 and 30 of the recovery period. For the chromosome count, three masses were randomly taken from the test tube each day, and 5–6 primary shoot primordia from each mass were used. A total of more than 100 metaphase cells was observed for each mass. In the untreated samples, all of the cells observed were diploid (Figs. 1C and 3A). Upon treatment with colcemid, the frequency of the diploid cells was greatly decreased, and the majority of the shoot primordia became mixoploid; on day 1.5 of the colcemid treatment, all but 1 were a mixoploid of di- and tetraploid cells (Fig. 3B), and on day 3, 16 out of 18 were a mixoploid of di-, tetra- and octoploid cells while the remaining shoot primordia were a mixoploid of di- and tetraploid cells (Fig. 3C).

The duration of the recovery period after release from the colcemid treatment markedly influenced the degree



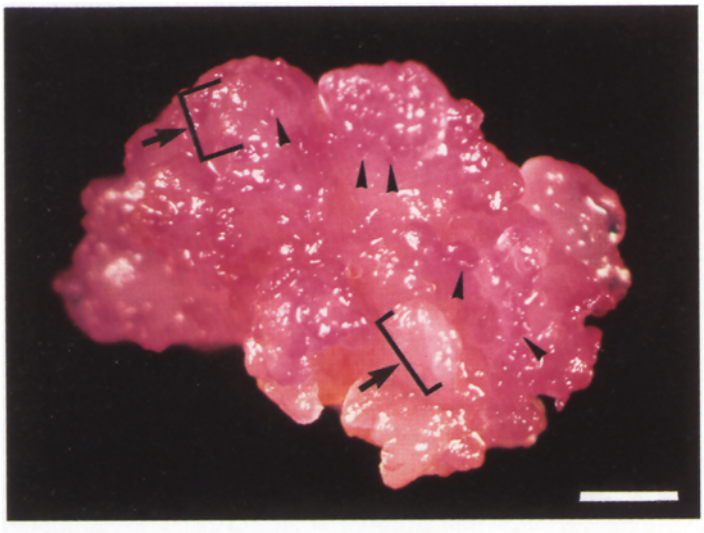


Fig. 2

Fig. 1A–F Diploid and tetraploid shoot primordia masses and the metaphase chromosomes. **A–C** Diploid, **D–F** tetraploid. **A, D** Masses subcultured in a test tube, **B, E** magnifications of a mass, **C, F** metaphase showing 2n = 4 and 2n = 8, respectively. *Bars* in **D, E** and **F** indicate 5 mm, 1 mm and 5 μm, respectively. **Fig. 2** A mass of shoot primordia stained by Feulgen's method. The primary shoot primordia, which consist of cells mitotically active, are stained dark pink (*arrowheads*); the secondary shoot primordia that develop from the primary ones show lower mitotic activity and are stained plae pink (*arrows*). *Bar*: 1 mm

of mixoploidy of the shoot primordia. On day 1.5 of the recovery period, the ploidy level of the shoot primordia was quite similar to that on day 0 of the recovery (= day 3 of the colcemid treatment; compare Fig. 3C and D). On day 3 of the recovery period, the majority of the shoot primordia were still mixoploid, but the frequency of those having octoploid cells was greatly decreased (Fig. 3E). On day 6 of the recovery period, shoot primor-

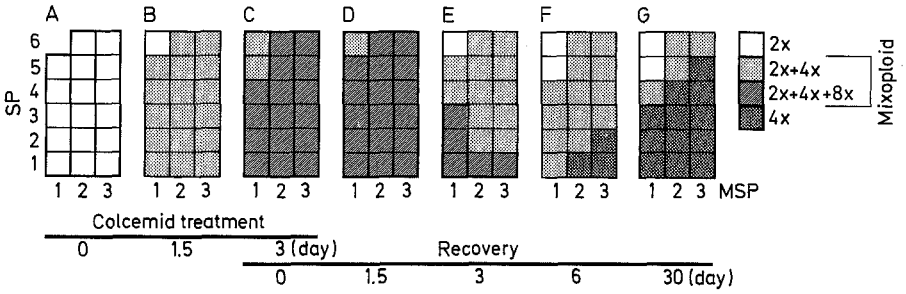


Fig. 3A–G Changes in the ploidy level of the shoot primordia in masses during and after the colcemid treatment. A *vertical column* and a *square* in the column represent the mass of shoot primordia and the shoot primordium, respectively. (*SP* Shoot primordia. *MSP* mass of shoot primordia)

dia that uniformly consisted of tetraploid cells appeared (Fig. 3F) and their frequency greatly increased on day 30, while the frequency of mixoploid shoot primordia greatly decreased (Fig. 3G). Thus, the frequency of tetraploid cells gradually increased in each shoot primordium as recovery period from the colcemid treatment was extended, but even after the 30-day recovery period none of the three masses was completely homogeneous tetraploid.

In order to obtain a completely non-chimeric tetraploid line in which all cells are solely tetraploid, isolation of the tetraploid shoot primordium was performed by cutting up the chimeric mass into separate shoot primordium domes. The shoot primordia masses, after a colcemid treatment of 3 days followed by a recovery period of 30 days, were cut into small pieces (approximately 1 mm in diameter), each of which had approximately one primary shoot primordium. The individual pieces were cultured as described in the Materials and methods. When each piece had developed into a mass approximately 10 mm in diameter, 1 month after cutting, six shoot primordia were randomly picked from each mass and then subjected to a chromosome count in the same manner as described above. The results are shown in Fig. 4.

All of the 15 masses propagated from the untreated shoot primordia consisted solely of diploid shoot primordia (Fig. 4A). Figure 4B shows the results obtained on the masses propagated from the pieces cut immediately after the 3-day colcemid treatment: 2 out of the 15 masses observed were homogeneous diploid, 10 were in a chimeric state of various combinations of ploidy level and only 3 were homogeneous tetraploid. Uniform octoploid shoot primordia were observed, but only in a chimeric state in 2 masses. Our efficiency in reducing the chimerism of the masses of the shoot primordia was much higher when they were cut into pieces after being allowed 30 days to recover from the colcemid treatment (Fig. 4C). Out of the 18 masses 7 were observed to be homogeneous tetraploid and 8 were diploid. The chimerism was found in only 2 masses. Cells with $2n = 7$, which was considered to be the result of an irregular mitosis, were also homogeneously observed in a mass. Thus, the homogeneous tetraploid masses were obtained at a frequency of 7/18, each of which could be isolated as a line of the tetraploid shoot primordia.

Next we examined the stability of the chromosome number in tetraploid shoot primordia artificially induced by the above experiment. Table 1 shows the results. A tetraploid line was established by subculturing a homogeneous tetraploid mass obtained after being cut into pieces 30 days after recovery from the 3-day colcemid treatment (Fig. 1D–F). The tetraploid mass propagated into comparatively larger masses during the subculture, having larger domes of the shoot primordia than those of the diploid ones (Fig. 1A, B). The chromosome number of the cells was examined after 98, 221 and 370 days of recovery. On day 98, all of the nine shoot primordia observed consisted uniformly of tetraploid cells. On day 221, five of the eight shoot primordia examined uniformly consisted of tetraploid cells while the others also had $2n = 8$ cells and a few other exceptions. On day 370, eight out of nine shoot primordia were uniformly of tetraploid cells. These results indicate that tetraploid shoot primordia artificially induced can maintain their chromosome number at a frequency above 90% for longer than 1 year.

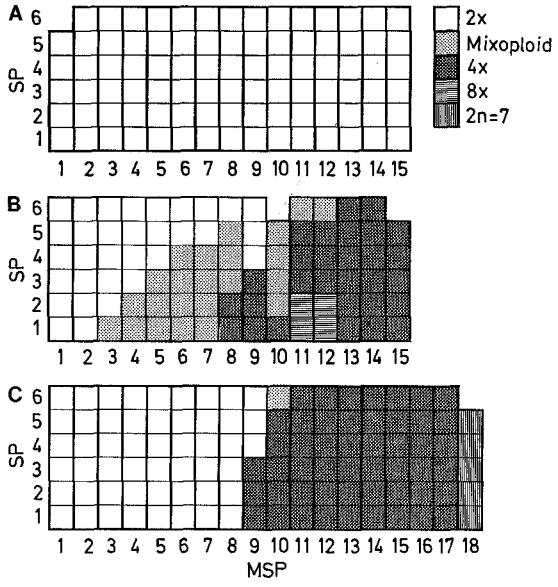


Fig. 4A–C The ploidy level of the shoot primordia 30 days after being cut into small pieces. A *vertical column* and a *square* in the column represent the mass of the shoot primordia and the shoot primordium, respectively. **A** Control without colcemid treatment, **B** shoot primordia cut immediately after colcemid treatment had been applied for 3 days, **C** shoot primordia cut after a 30-day recovery period from the 3-day colcemid treatment

Table 1 The chromosome number of cells from an isolated line of tetraploid shoot primordia of *Haplopappus gracilis*

Total days after the colcemid treatment	Code number of shoot primordium	Number of cells with the following chromosome number				
		7	8	8+1f[a]	16	Total
	1-1[b]		6			6
	1-2		8			8
	1-3		9			9
	1-4		11			11
98	1-5		12			12
	1-6		15			15
	1-7		16			16
	1-8		20			20
	Total		97			97
	1-1		9			9
	1-2		12			12
	1-3		8		3	11
	2-1		10			10
221	2-2		6	1		7
	3-1		9			9
	3-2		10			10
	3-3	1	4			5
	Total	1	68	1	3	73
	1-1		8			8
	1-2		16			16
	1-3		17			17
	2-1		7			7
	2-2		8			8
370	2-3		19			19
	3-1		10			10
	3-2		12	1		13
	3-3		16			16
	Total		113	1		114

[a] A chromosome fragment
[b] The left and right numbers represent the code of the mass and the shoot primordium in the mass, respectively

Regenerated plants from tetraploid shoot primordia were obtained. The chromosome number of the cells in the shoot tips was examined in ten regenerated plantlets. Five to 12 metaphase cells were examined in each shoot tip, and all of them were $2n = 8$.

Discussion

Chimeras are considered to negatively affect breeding performance with cultured tissue except when these chimeric traits are specifically needed. In the investigation reported here we obtained results showing an efficient method for reducing chimerism. The tetraploid cells induced by the colcemid treatment were propagated more predominantly through mitosis than cells of other ploidies. The frequency of these tetraploid cells gradually increased in most of the shoot priomordia during the recovery period. Shoot primordia consisting uniformly of tetraploid cells were observed in the prolonged recovery period, but chimerism still remained after a 30-day recovery at the mass level: most of the shoot primordia were uniformly tetraploid in each mass, while a few diploid and mixoploid shoot primordia were still observed in the same mass. Therefore, we made an attempt to isolate the completely homogeneous tetraploid mass propagated from the tetraploid shoot primordium by cutting the chimeric mass into small pieces.

The effect of cutting up a mass is evident when the results of the chromosome count on the samples used in Fig. 4B and Fig. 3G are compared. Both samples were actually fixed after a 30-day recovery from the 3-day colcemid treatment, with and without cutting, respectively. Both data show that the ploidy level of each shoot primordium is similar. However, in Fig. 3G (without cutting) none of 3 masses were homogeneous, while in Fig. 4B (with cutting), complete tetraploid and diploid masses were obtained, even though the efficiency was much lower than the result shown in Fig. 4C.

Non-chimeric tetraploid masses were most efficiently isolated by cutting a mass that had been allowed to recover for 30 days, when most of the shoot primordia were uniformly tetraploid. The tetraploid masses of the shoot primordia artificially induced by this method could maintain chromosomal stability for at least 1 year, and while this illustrates the "chromosomal" stability of the shoot primordia, it is not fully enough to prove "genetic" stability in the strictest sense; even so, part of the criteria are met.

The present results also support the histological results observed by Tanaka and Ikeda (1983): "A new shoot primordium developed from a few cells". Each shoot primordium just after the colcemid treatment was absolutely cytochimera, in a mosaic state with half the cells diploid and the other half tetraploid (Tanaka et al. 1988b). After the recovery period, the tetraploid cells could propagate into domes that consisted uniformly of tetraploid cells, presumably because the number of cells participating in the initiation of new shoot primordium was very small. The process of initiation of new shoot primordia may also be similar to that of the meristemoid (Torrey 1966; Thorpe and Murashige 1970; Nessler 1982; White 1984) and meristematic nodule (Jones 1974; Pareek and Chandra 1978) from callus or suspended cells. In fact, masses of shoot primordia have been regenerated from suspended cells that were induced from shoot primordia (Tanaka et al. 1985). Furthermore, non-chimeric masses of shoot primordia with a karyotype mutation have been induced from suspended cells of *Crepis capillaris* ($2n = 6$) that were derived from hypocotyl callus (Taniguchi and Tanaka 1989).

The process of reducing chimerism through shoot primordia propagation is also similar to the case in which regenerated plants induced from calli had less chimerism although the original calli were chimerical (Mitra et al. 1960; Murashige and Nakano 1966; Sacristán and Melchers 1969; Mehra and Mehra 1974; Hermsen et al. 1981; Broertjes and van Harten 1985). Pierik (1987) suggests in his review that the "development of

adventitious shoots" is important if complete mutants are desired after mutation induction, because adventitious shoots usually originate from a single cell. His suggestion can be applied exactly in our case, with respect to subsequently obtaining non-chimeric plant individuals.

It is of great advantage to have a system of tissue culture by which mutants can be stably maintained, especially in species of annual and outbreeding plants. Shoot primordia are considered to be among the most useful of materials for the induction and preservation of mutants: they can be rapidly mass-propagated without further differentiation, keeping their chromosomal stability for a long period. The problem remaining to be solved is the number of cells participating in the initiation of a new shoot primordium. We could not establish that a shoot primordium dome is really originate from only a very few cells by the present experiment using the colcemid treatment because many tetraploid cells, which had cytogenetically the same appearance, must have been simultaneously induced all around the mass. This question will be solved when cytochimeras, induced by chemical treatment or irradiation which causes at-random changes in the chromosome structure, can be separated as non-chimeric mutant lines of the shoot primordia.

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