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Chromosome Number Variation of Callus Cells and Regenerated Plants in *Asparagus officinalis* L.

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Chromosome number of the plants regenerated directly from lateral bud and shoot tip of asparagus (*Asparagus officinalis* L.) was stable ($2n=20$). In callus cells induced from lateral bud on MS medium supplemented with 10 μM NAA and 1 μM BA, chromosome number varied widely and the range of the variation differed with the explants used. By continuing the callus culture, chromosome number in GK and 56×22p callus cells had a tendency to increase still more. GK and 56×22p calli included plenty of middle and large-sized callus cells in addition to small-sized cells. In some callus cells, multiple nuclei were observed and as is expected to occur in irregular cell division, which is surmised to be one of the causes which leads to the increase in chromosome number. Most of the plants regenerated from 56×22p callus were normal in chromosome number, but some aneuploid and polyploid plants were regenerated. While, in MW500W calli, marked increase of chromosome number was not observed and these small cells divided vigorously. All of the plants regenerated from MW500W callus showed normal chromosome number ($2n=20$).

Introduction

Recently, asparagus (*Asparagus officinalis* L.) has been propagated by tissue culture (single nodal segment culture, somatic embryogenesis *etc.*) commercially. It is very important to investigate the genetic stability in the regenerating process from the explants used. The genetic variability or instability of callus cells is well characterized by the variation in chromosome number[1]. Variability of chromosome number and morphology in callus cells was reported in a large number of plants. In asparagus, however, there were few reports on the chromosome number variability of callus cells and the relation between its variation and chromosome number of regenerated plants. In the present paper, the stability of chromosome number in regeneration without callus formation, and the variation of chromosome number in subcultured callus cells and regenerated plants were examined.

Materials and Methods

1. *Plant regeneration from lateral bud and shoot tip*

Stock plants of 3 asparagus cultivars (*Asparagus officinalis* L., $2n=20$) maintained at Experimen-

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tal Farm of Hokkaido University (Sapporo city), Grüne krone (GK) (plant No. 1), 56×22p (plant No. 1, 2, 3, 31) and MW500W (plant No. 19), were used in the present experiment. Spears of these plants were surface-sterilized with 70% ethanol for 10 seconds and 1% sodium hypochlorite for 15 minutes. They were rinsed 2 times in sterile distilled water.

Some lateral buds were excised and placed on Murashige & Skoog (MS) medium supplemented with 10 μM IBA and 0.05 μM BA for plant regeneration without callusing. Further, 2 mm-shoot tips of plants regenerated from lateral buds were transferred to new medium for proliferation and a large number of regenerated plants were obtained. Chromosome numbers in the root tips of the regenerated plants were observed.

On the other hand, some lateral buds were placed on MS medium supplemented with 10 μM NAA and 1 μM BA for callus induction. Induced calli were transferred to the same medium on which callus was induced after 2 months. Thereafter, proliferated calli were subcultured on new medium every month. Then, some regenerated plants were obtained by transferring those calli to MS medium supplemented with 2.5 μM IAA and 4.4 μM BA. In the process, the chromosome number and the number of nuclei were observed in the 2nd-subcultured calli, 5th-subcultured calli and regenerated plants.

All media contained 7 g/l agar and 20 g/l sucrose, and pH was adjusted to 5.7 prior to autoclaving. These calli were cultured at 25°C under a 16/8-hour day/night photoperiod and illuminated with 3,000-4,000 lux provided by fluorescent lamps.

2. *Observation of chromosome number*

Induced calli and root tips of regenerated plants were stored at 0°C for 24 hours and fixed in karunoy-fluid (ethanol : acetic acid=3 : 1) for 24 hours. They were stained with aceto-carmine for 1 hour, and their chromosome numbers were investigated by the squash method.

Results and Discussion

1. *Chromosome number of the plants regenerated without callusing*

After 5 weeks of incubation, about 25% of the lateral buds used developed into plantlets in the 3 cultivars used. The normal chromosome number ($2n=20$) was observed in all of them (**Table 1**). All of 2 mm-shoot tips developed into plantlets and all plants regenerated through shoot tip-subculture showed $2n=20$. It was recognized that chromosome number of the plants regenerated

Table 1. Chromosome number observed in the plants regenerated from lateral shoot apices and propagated through 2 mm-shoot tip culture.

Explant	Cultivars used	Plant No.	No. of plants observed	No. of plants with normal chromosome number ($2n=20$)
Lateral bud	56×22p	2	5	5
		3	5	5
		31	5	5
	MW500W	19	5	5
	GK	1	5	5
Shoot tip	56×22p	2	5	5
		3	8	8
		31	8	8
	MW500W	19	5	5
	GK	1	3	3

without callusing was stable ($2n=20$) in asparagus. It was reported that there was no variability in chromosome number of the plants regenerated directly from shoot apices and lateral buds in other plants[2].

2. *Chromosome number variation of callus cells*

In 2nd-subcultured calli of the 3 cultivars used, which were friable, a large variation was observed in chromosome number and a high frequency of ploidy was recognized (**Fig. 1, 2**). Especially, normal chromosome number ($2n=20$) was observed with the highest frequency. The variation range of chromosome number was different among the cultivars used. In GK calli, chromosome number varied from 7 to 50 with 47% (85/179) of callus cells distributed close to $2n=20$, between 17 and 23. In 56×22p calli, chromosome number varied from 5 to 54 with $2n=10$-20 observed in 75% (109/144) of callus cells. On the other hand, for MW500W calli, the variation range in chromosome number was smaller than those of the 2 previous cultivars with the chromosome number distributed between 9 and 33.

In 5th-subcultured calli, the variation range in chromosome number spread widely in GK and 56×22p calli, and callus cells with a chromosome number of more than 100 were observed (**Fig. 2**). In addition, in GK calli, the frequency of $2n=20$ was 18% (13/70) of the callus cells and higher than that of 2nd-subcultured calli. The frequencies of ploidy ($2n=10, 20, 40$) were higher than those of other chromosome numbers in 56×22p calli. On the other hand, in MW500W calli the variation range of chromosome number was similar to that of 2nd-subcultured calli. It varied from 7 to 31

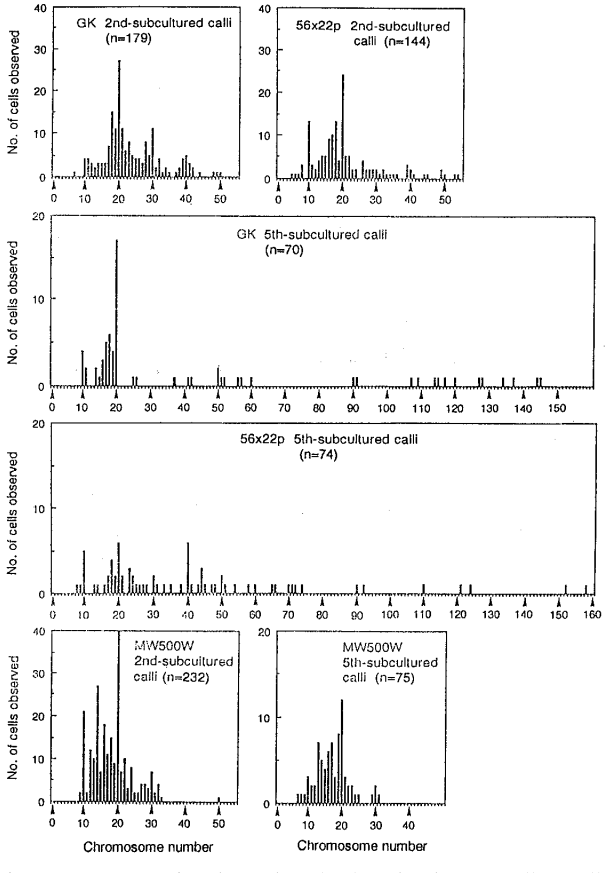

Fig. 1 Variation of chromosome number in 2nd and 5th-subcultured callus cells.
(n) in each graph indicates the total number of callus cells observed the chromosome number.

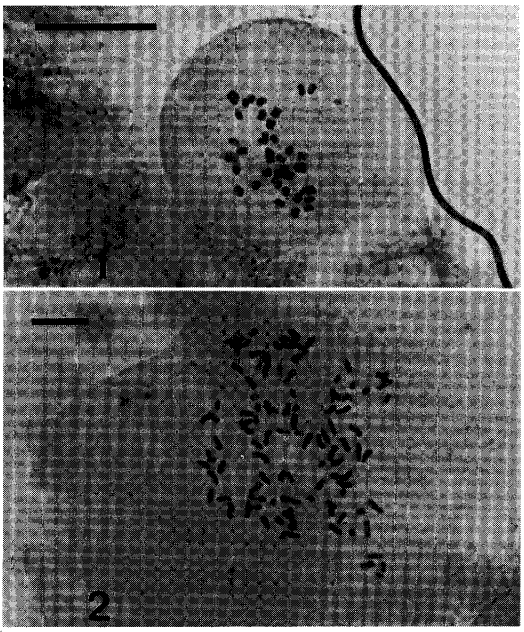

Fig. 2 Examples of callus cell with aneuploid-chromosome number observed in 5th-subcultured GK calli. Scale bar indicates 20 μm. Chromosome number, 1 ; $2n=37$, 2 ; $2n=107$

Table 2. Relation between the cell size and the chromosome number observed in the 5th-subcultured GK calli.

Cell size		No. of cells observed																								
Grade	Average (μm)	Chromosome number																								
		10	11	14	15	16	17	18	19	20	25	26	37	41	50	51	56	57	90	107	114	117	128	137	144
Small	15	2	1	1	1	1	3	3	1	9	1	1													
Middle	50												1	1	1	1	1	1							
Large	200																		1	1	1	1	1	1	1

and the frequency of normal chromosome number (16%, 12/75 callus cells) was higher than that of any other chromosome number.

Callus cells with a high ploidy level was observed in tobacco[3,4], wheat[5], sugarcane[6] and other plants[7]. Chromosome number in callus cells increased as the culture period became longer in a large number of plants[3,8,9]. Besides, in sugarcane, each clone had a different chromosome population mode[6]. MW500W (plant No. 19) calli is assumed to be a clone in which the chromosome number does not increase by subculture.

3. *Interaction among size of callus cell, nucleus number and chromosome number*

GK callus cells which had a large range of variation in chromosome number were classified into 3 grades (small, middle, large) by their size (**Table 2**). The small-sized cell was approximately 15 μm in diameter and sphere in shape. Vigorous cell division was observed and chromosome number varied from 10 to 26 in small sized-cells. The middle-sized cell was approximately 50 μm in size and egg-shaped. Chromosome number of $2n=37$-57 were observed in middle-sized cells. The large sized -cell was approximately 200 μm in diameter and egg-shaped or elongated egg-shape. A large chromosome number, $2n=90$-144, was observed in large sized-cells and chromosome number increased according to the extension of cell size. MW500W callus consisted of small-sized cells.

While all 3 grades, especially middle and large, were observed in GK and 56×22p calli.

Multiple nuclei were observed in middle-sized and large-sized cells. The number of nuclei tended to increase according to the subculture duration. Especially, multipe nuclei were observed in GK and 56×22p 5th-subcultured callus cells (**Fig. 3, 4**). These cells were egg-shaped or elongated egg-shape. There was a variation in nucleus size observed (data not shown here). On the other hand, MW500W calli consisted of a large number of sphere or egg-shaped small sized-cells with vigorous cell division. Almost all of MW500W callus cells had a single nucleus.

Multi-nucleate cells were also observed in suspension cultured cells of sugarcane and they indicated the possibility of high polyploidy[6]. Irregular cell-division, such as nuclear division without cell division, is expected to occurr in callus cells with nuclei multiplication.

4. *Chromosome number of the plants regenerated from callus*

Normal chromosome number ($2n=20$) was observed in all plants regenerated from MW500W calli (**Table 3**). On the other hand, though most of plants regenerated from 56×22p calli showed $2n=20$, 2 aneuploids and 2 polyploids ($2n=16, 24, 30$) were differentiated and plantlets with extreme aneuploid were not regenerated.

It is well known that normal chromosome number was shown in the regenerated organs though there were many polyploid and aneuploid in callus cells in the previous reports[10-12]. Regarding the chromosome number of regenerates, the different types may be classified into 3 groups: diploid, polyploid and aneuploid, and mixploid or chromosome number chimera. It is reported that the chromosome constitution of regenerates seems to be as highly dependent on species , kind of

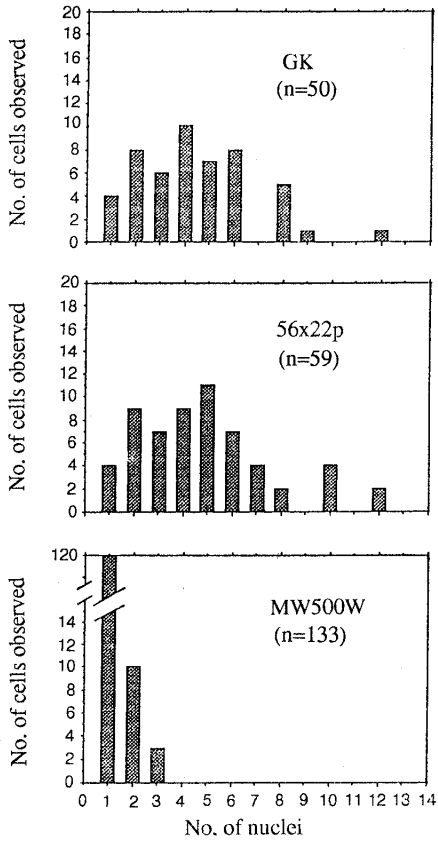

Fig. 3 Variation of nucleus number in 5th-subcultured GK callus cells.

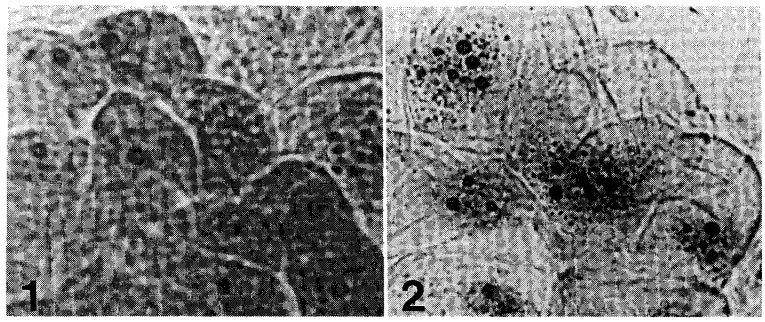

Fig. 4 Single(1) and multiple nuclei(2) observed in 5th-subcultured GK callus cells.

Table 3. Chromosome number observed in the plants regenerated from the subcultured calli.

Cultivars used	No. of regenerated plants observed	No. of plants classified by chromosome number			
		16	20	24	30
56×22p	21	1	17	1	2
MW500W	20	0	20	0	0

original explant, or conditions of regeneration from callus and so on. From our observation, the chromosome number of regenerates was effected by the cell division in the callus. Namely, MW500W callus had numerous small sized-cells which divided vigorously. While, there was a probability of irregular or non-vigorous cell division in 56×22p callus cells because of middle and large cell size. It was assumed that the chromosome number of the regenerated plants may be related closely to the cell division of callus cells. Further work is needed to define whether or not chromosome number chimera exists in the aneuploid plants ($2n=16$ and $2n=24$).

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《和文要約》

アスパラガスにおけるカルス細胞と再分化個体の染色体数の変異

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アスパラガスの腋芽と2 mmの茎端より再生した個体の染色体数はすべて$2n=20$で安定していた．腋芽より誘導したカルス細胞の染色体数は供試した3品種・系統とも変異を示したが，変異の幅は供試系統により異なった．GKと56×22pのカルスの染色体数は10以下から50以上に変異したのに対し，MW500Wのカルスは$2n=10$～30の変異であった．どの系統も倍数性の値が多く出現する傾向が認められた．継代培養を続けることによりGKと56×22pでは変異が大きく拡大し，100以上の染色体数も観察された．染色体数の多い細胞は大きく，核数も増加しており，不正常な細胞分裂の可能性が示唆された．他方，MW500Wでは継代培養による染色体数変異の拡大は認められず，ほとんどが1核で，旺盛に分裂する小さな細胞が多かった．この様なカルスからは2倍体($2n=20$)の個体のみ再分化したが，大幅な染色体数変異を示し，大型の細胞が多く含まれていた56×22pのカルスからは異数体も出現した．