Original Papers Plant Tissue Culture Letters, 8(2), 67~72(1991)

Number of Chloroplasts in Haploids and Diploids Produced via Anther Culture in *Brassica campestris*

Yoh HAMAOKA*, Yukio FUJITA** and Sumio IWAI*

* *Applied Plant Research Laboratory, Japan Tobacco Inc.*, 1900 Idei, Oyama, Tochigi 323, Japan
** *Tohoku Seed Co., Ltd*, 1625 Nishihara, Himuro, Utsunomiya, Tochigi 321-32, Japan

(Received September 29, 1990)
(Accepted December 19, 1990)

Microspore-derived plants were produced via anther culture in *Brassica campestris*. The cytological analysis of these plants revealed the existence of diploids, tetraploids as well as haploids. In these plants, the number of chloroplasts in guard cells were clearly related to the ploidy level. The number of chloroplasts in mesophyll protoplasts was also related to the ploidy level, though the relationship was not as clear as in guard cells. The number of chloroplasts in both types of cells increased after the number of chromosomes was artificially doubled with colchicine.

Introduction

Anther culture has been employed in many species to obtain microspore-derived plants, which could be of great value for breeding and the study of genetics[1]. Usually, cultured anthers of *Brassica campestris* yield haploid, diploid and polyploid plantlets. Thus, determination of ploidy level and chromosome doubling of haploids are essential steps for the application of anther culture to plant breeding.

The common method for determining ploidy level is chromosome counts of root tip cells or pollen mother cells. However, these methods are laborious and time consuming when many plants are to be analyzed. On the other hand, some morphological characteristics, such as leaf length, width, anther length and stomatal length, have been reported to be related to the ploidy level in several plant species. The number of chroroplasts in guard cells is one of these characteristics and is clearly related to the ploidy level in *Solanum*[2], Petunia[3][4], Glycine[5], *Gossypium*[6] etc. However, it remains uncertain whether the relationship between the number of chloroplasts and the ploidy level is specific to guard cells or not. In the present study, we have produced microspore-derived plants via anther culture, and investigated the relationship between the ploidy level and the number of chloroplasts in guard cells as well as in mesophyll protoplasts.

Materials and Methods

Plant material and anther culture : *Brassica campestris* L. ssp. *pekinensis* cv. "Tsubame" (Tohoku Seed Co.) was used for all the experiments in this report. Seeds were sown in potted soil in a greenhouse. Seedlings with their cotyledons just unfolded (about a week later) were transferred to a cold room (5-7°C), and kept there for 30-40 days. Then they were grown in a greenhouse conditioned at 17-25°C. Flower bud selection and anther culture were performed as described by

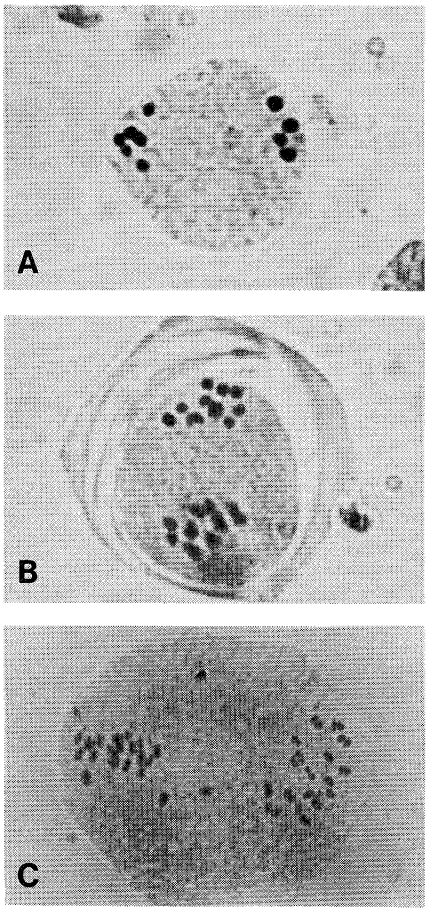

Fig. 1 Haploid (A), diploid (B) and tetraploid (C) pollen mother cells at anaphase I. The PMC of the haploid shows a 4-6 univalent separation while the diploid and tetraploid show 10-10 and 20-20 separation, respectively.

Keller and Armstrong (1979)[7]. The embryoids emerged from the cultured anther were transferred to B5 medium[8] containing 0.02 mg/*l* NAA, 1.2 mg/*l* BA and 2% sucrose (shooting medium). As shoots developed, they were transferred again to hormone-free B5 medium to induce root formation. They were then potted in vermiculite, placed under mist for two weeks, transplanted and kept in a greenhouse.

Chloroplast number: The epidermis was peeled from the lower surface of the leaves of anther-derived plants, mounted in distilled water, and observed under a fluorescence microscope (Olympus BH2-RFK with a filter combination of BP490 and 0515).

The autofluorescence of chloroplasts was detected and the number of chloroplasts in guard cells was counted. Twenty cells were counted for each plant.

Mesophyll protoplasts were isolated from the leaves of anther-derived plants. The leaves which had just fully expanded were cut into small pieces, placed in an enzyme mixture (0.8% Cellulase Onozuka RS, 0.1% Pectolyase Y23, 0.55 M mannitol, 20 mM $MgCl_2$) and incubated for 3 h at 30°C. The enzyme solution containing protoplasts was filtered through a nylon mesh (60 μm pore size), centrifuged at 500 rpm for 5 min and then the pellet was washed twice with a mannitol solution (0.55 M mannitol, 20 mM $MgCl_2$). The density of the protoplasts was adjusted to ca. 10^5/m*l*, and the number of chloroplasts in the protoplasts was counted under fluorescence microscope as described

Table 1. Embryogenesis in cultured anther of *Brassica campestris*.

No. of anthers cultured	No. of anthers producing embryoids	Frequency (%)	No. of embryoids	Efficiency[1]
284	129	45.4	318	1120

(1) Expected number of embryoids from 1,000 anthers

above. Twenty cells were counted for each plant.

Cytological analysis : The number of chromosomes was determined in pollen mother cells (PMC) at anaphase I after fixation in Carnoy's solution (ethanol : acetic acid : chloroform, 6 : 3 : 1) and staining with aceto-orcein.

Artificial doubling of chromosome number : The chromosome doubling of haploids was carried out by treating a terminal bud with colchicine. A small, cotton wool plug containing 0.1% aqueous colchicine solution was applied for 24 h to the terminal buds[9].

Results and Discussions

Microspore-derived plants were produced via anther culture in *B. campestris*. The efficiency with which microspore-derived plants were obtained was relatively high (ca. one plant from one cultured anther) for this variety (Table 1). The embryoids emerged were transplanted to shooting medium, where the majority (approximately 80%) of them produced normal shoots. They easily produced roots in B5 hormone free medium. They were then potted and grown in the greenhouse.

The PMC analysis of these plants revealed the existence of diploids, tetraploids as well as haploids (Fig. 1). Among the 31 plants examined, there were 15 haploids, 15 diploids and 1 tetraploid.

The number of chloroplasts per guard cell (C/GC) was counted for each ploidy. The average number of C/GC in 10 haploids and 10 diploids are shown in Fig. 2. Generally, the haploids had an average of 2-4, while the diploids had 4-6. The differences among cells of a single plant and among

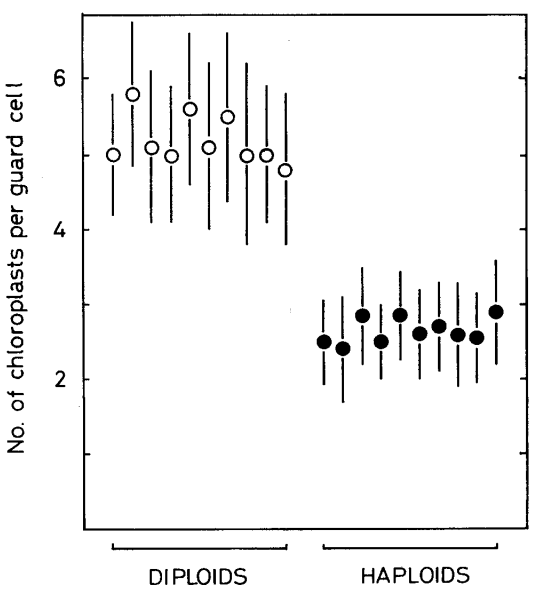

Fig. 2 Average number of chloroplasts per guard cell in 10 haploids (●) and 10 diploids (○). Each point represents an average of 20 cells. Bars indicate standard deviations.

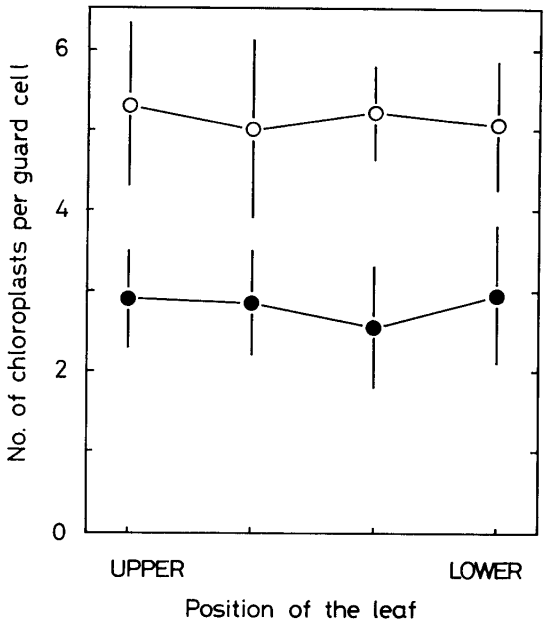

Fig. 3 Number of chloroplasts in guard cells of leaves at different stages of development. Four haploid (●) and four diploid (○) leaves at different stages were investigated. Each point represents an average of 20 cells. Bars indicate standard deviations.

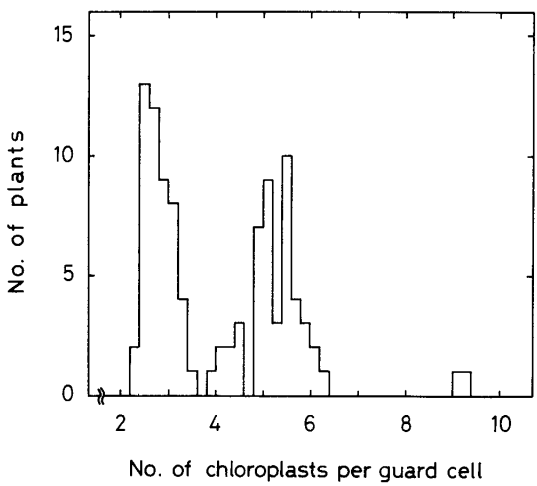

Fig. 4 Average number of chloroplasts per guard cell in plants produced via anther culture.

plants of the same ploidy were not significantly different. C/GC was stable with the aging of the leaf, showing no significant difference among leaves at different stages of development (Fig. 3). Fig. 4 shows the distribution of the average C/GC in 98 plants produced via anther culthre. They were divided into three groups: 2-4, 4-6 and 8-10. These groups corresponded to haploid, diploid and tetraploid respectively, though a few exceptions were observed.

The number of the chloroplasts in mesophyll protoplasts (C/PP) was also different for haploids and diploids, though the deviations of the chloroplast number were greater in mesophyll protoplasts than in guard cells (Table 2).

By treating terminal buds with colchicine, the chromosomes of about half of the treated haploids doubled in number, while the rest remained haploid, died or became tetraploid. The C/GC and C/PP in one plant were counted both before and after the chromosome doubling. In both types of cells, the number of chloroplasts increased about two fold after chromosome doubling (Table 3).

Table 2. Number of chloroplasts in mesophyll protoplasts

Ploidy	C/PP
Haploid	23.4±8.0a[1)]
Diploid	34.3±9.6b

100 cells from 5 plants (20 cells/each plant) were counted in each ploid. Data were shown as the average ± standard deviation.

1) Different letters indicate statistical significance of the differences between the mean values at 1% level.

Table 3. Number of chloroplasts in guard cells (C/GC) and mesophyll protoplasts (C/PP) before and after the chromosome doubling.

	C/GC	C/PP
Before treatment	2.4±0.7a[1)]	19.5±4.0a[1)]
After treatment	6.4±2.0b	33.3±14.6b

Data were shown as the average ± standard deviation.

1) Different letters in a column indicate statistical significance of the differences between the mean values within a column at 1% level.

C/GC was closely related to ploidy level in anther-derived *B. campestris*, and stable with the age of the plant. Therefore this characteristic can be a useful parameter for determining the ploidy level. In fact, we were able to determine the ploidy of the plants from their C/GC (Fig. 4). C/PP was also related to the ploidy level. However, the relationship was not as reliable as for C/GC. The protoplasts isolated from the leaves are a mixture of various types of cells from spongy parenchyma and palisade parenchyma tissues. This heterogeneity of the protoplast population might explain the indistinct relationship between C/PP and ploidy level. Both C/GC and C/PP increased after artificial chromosome doubling with colchicine. These data suggest that not only in guard cells but also in other cells, the number of chloroplasts is determined by the ploidy level or genome size.

References

1) Maheshwari, S. C., A. Rashid, A. K. Tyagi, 1982. Amer. J. Bot., **69** : 865-879.
2) Pehu, E., R. E. Veilleux, K. W. Hilu, 1987. Amer. J. Bot., **74** : 47-52.
3) Mitchell, A. Z., M. R. Hanson, R. C. Skvirsky, F. M. Ausubel, 1980. Z. Pflanzenphysiol., **100** : 131-146.
4) Santos, R. F., W. Handro, 1983. Theor. Appl. Genet., **66** : 55-60.
5) Beversdorf, W. D., 1979. Can. J. Plant Sci., **59** : 945-948.
6) Chaudhari, H. K., J. R. Barrow, 1975. Crop Sci., **15** : 760-763.
7) Keller, W. A., K. C. Armstrong, 1979. Theor. Appl. Genet., **55** : 66-67.
8) Gamborg, O. L., R. A. Miller, K. Kojima, 1968. Exp. Cell Res., **50** : 151-158.
9) Wenzel, G., F. Hoffmann, E. Thomas, 1977. Z. Pflanzenzuchtg, **78** : 149-155.

《和文要約》

葯培養により作出したハクサイ花粉由来植物の，孔辺細胞及び葉肉プロトプラスト中の葉緑体数

浜岡　陽*，藤田幸雄**，岩井純夫*

*日本たばこ産業株式会社植物開発研究所
**トーホク株式会社清原育種農場

ハクサイ（*Brassica campestris* ssp. *pekinensis*）の花粉由来植物を葯培養により作出しその染色体数を調査したところ，半数体，二倍体，そして少数の四倍体が混在していた．このうち，半数体と二倍体に関して，孔辺細胞の葉緑体数（C/GC）を調査した結果，両者の間に有意な差が認められた．また，C/GC は植物の加齢や生育環境に対して安定していた．従ってこの値はハクサイの倍数性を推定する指標として用い得ると考えられる．一方，葉肉プロトプラストの葉緑体数（C/PP）も，C/GC に比べてばらつきは大きいが，半数体，二倍体間で有意な差があった．また，半数体をコルヒチン処理によって倍加すると，C/GC, C/PP ともほぼ倍に増加した．これらの結果は，孔辺細胞以外の細胞においても葉緑体数がゲノムの倍数性によって支配されていることを示している．