体細胞雑種カルスにおける葉緑体ゲノムの分離様式に関する研究(3)

誌名	The Japanese journal of genetics
ISSN	0021504X
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巻/号	61巻5号
掲載ページ	p. 437-445
発行年月	1986年10月

Jpn. J. Genet. (1986) **61**, 437-445

Studies on the mode of separation of chloroplast genomes in parasexual hybrid calli
III. Random separation of two types of chloroplast genomes in a hybrid callus

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(*Received August* 21, 1986)

ABSTRACT

In the parasexual hybrid callus between *Nicotiana glauca* and *N. langsdorffii*, the two parental types of chloroplasts separate during callus development. The system to investigate the mode of separation involves the hormone independent growth of the hybrid callus between *N. glauca* and *N. langsdorffii* as a selective marker for the parasexal hybrid and the isoelectrofocusing patterns of the large subunit (*LS*) of Fraction I protein as a chloroplast genetic marker. We improved this system by utilizing *N. gossei* as a chloroplast donor, because the isoelectrofocusing patterns of *LS* from *N. gossei* and that from *N. langsdorffii* can be distinguished clearly. The hormone independency which is suitable for selecting hybrids in the state of small calli was restored by transmitting the nuclear genome of *N. glauca* into *N. gossei* by means of sexual hybridization. Using the improved system, the mode of chloroplast separation was investigated in more detail. It was found that 84% of the cells in a two month old parasexual hybrid callus have only one or the other parental type of chloroplasts and 16% of the cells have two types.

We calculated the rate of separation using the formulae based on the assumption that the two types of chloroplasts separate at random by cell divisions in the callus. The result obtained by the calculation turned out to be consistent to the experimental data obtained by the analysis of the two month old parasexual hybrid callus, showing that our assumption is reasonable.

1. INTRODUCTION

There are many important genes for photosynthesis encoded by chloroplast genome (Rochaix 1985), so that the improvement of the function of photosynthesis may be achieved by changing the chloroplast genes. However, so far it is difficult to accomplish this program. Some of the reasons for this difficulty are: (1) In most of higher plants, a chloroplast is maternally inherited and thus there is little chance for two different types of chloroplasts to be mixed in one cell (Possingham 1980; Tilney-Bassett 1981). (2) There is

a high copy number of chloroplast DNA molecules in any given chloroplasts, which makes it more difficult for any mutant DNA molecules to be selected and expressed (Lamppa 1979). (3) Vectors suitable for chloroplast transformation have yet to be discovered.

One way to overcome these obstacles is to make parasexual hybrids so that two different types of chloroplasts may be mixed in a fused cell. This in turn enhances the possibility of inducing interspecific chloroplast hybridization. A recent report described about an interspecific chloroplast recombination in a *Nicotiana* parasexual hybrid (Medgyesy *et al.* 1985).

However, there have been many reports stating that although some of the parasexual hybrid plants regenerated from fused cells have two types of chloroplasts, the majority of the plants have only one type (Chen *et al.* 1977: Schiller *et al.* 1983; Glimelius *et al.* 1981). In our previous reports, we investigated how two types of chloroplasts segregate during the propagation of cells in the somatic hybrid callus between *Nicotiana glauca* (*G*) and *N. langsdorffii* (*L*). We found that, although the two types of chloroplast had been initially mixed in the fused cells, after two months, they were separated substantially (Akada *et al.* 1983: Akada and Hirai 1983).

In these papers, we used the large subunit (*LS*) of the Fraction I Protein (Ribulose-1, 5-bisphosphate carboxylase oxygenase EC 4.1.1.39: FIP) as a chloroplost genetic marker. The *LS* bands from *G* are focused at higher pH than those from *L* in an isoelectrofocusing gel containing 8M urea. However, it is difficult to distinguish the former from the latter. The *LS* bands from *N. gossei* are focused at still higher pH than those from *N. glauca* when the FIPs are carboxymethylated. Therefore the pH difference between *N. gossei* and *N. langsdorffii* is larger than that between *N. glauca* and *N. langsdorffii*. It will be better to utilize this system provided that the hormone independent growth of the hybrid callus between *N. glauca* and *N. langsdorffii* which is also necessary for selecting a parasexual hybrid in the state of a small callus can be restored in this system.

This paper describes how we improved the system to investigate the mode of separation of chloroplasts. Using this system, we found that the mode of separation follows the mathematical estimation based on random separation.

2. MATERIALS AND METHODS

Plant materials

Seeds of *Nicotiana glauca, N. langsdorffii* and *N. gossei* were obtained from Japan Tobacco INC. In order to utilize the selection system for the parasexual hybrid between *N. glauca* and *N. langsdorffii*, the nuclear genomes of *N. glauca* were transmitted into *N. gossei* by sexual hybridization. *N. gossei* was sexually crossed by *N. glauca* and the hybrid F_1 (♀ *N. gossei* × ♂ *N.*

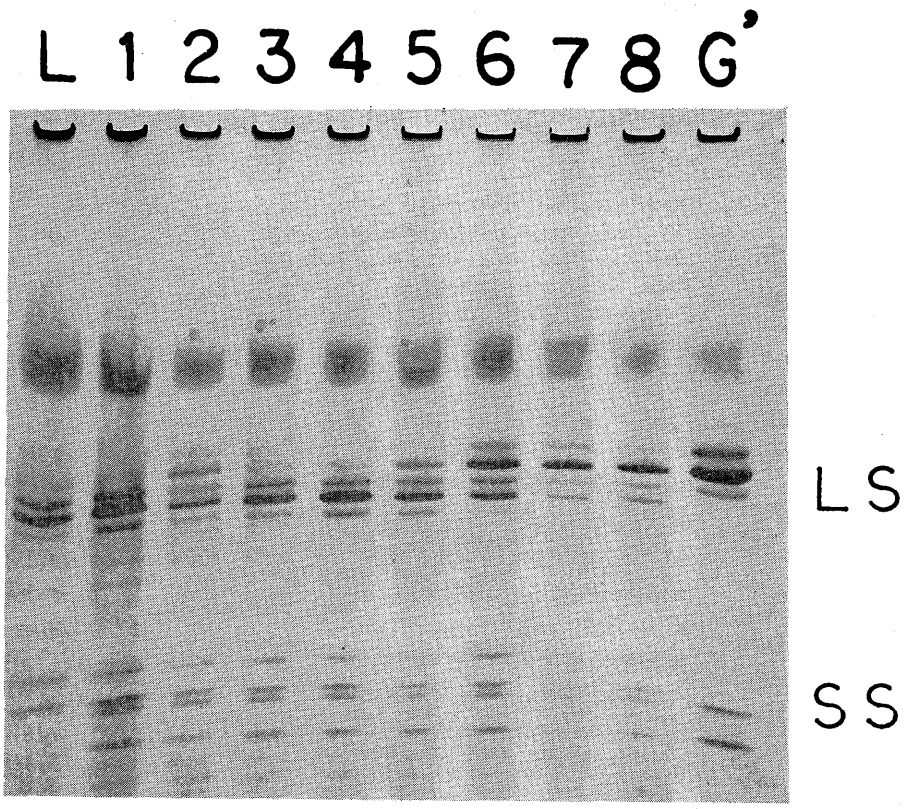

Fig. 1. The isoelectrofocusing of FIPs isolated from *G'*, *N. langsdorffii* (*L*) and the parasexual hybrid calli (1–8).

glauca) was backcrossed by *N. glauca*. The offspring produced by this backcross [♀ (♀ *N. gossel* × ♂ *N. glauca*) × ♂ *N. glauca*] was designated as *G'*.

Protoplast fusion and cell culture

Protoplasts from *G'* and *L* were mixed, treated with PEG and cultured in the Murashige-Skoog liquid medium (Murashige and Skoog 1962) supplemented with 3 mg/ml of 2,4-D and 0.5 mg/ml of kinetin as previously reported (Akada *et al.* 1983) Two weeks after the protoplast fusion, the culture medium was replaced by the hormone free Murashige-Skoog medium. One month after the fusion, the green colonies that had been regenerated from the fused cells were transferred to the hormone free agar medium.

Isoelectrofocusing of FIP

The FIP was isolated from leaves of *G'*, *L* and the green calli and was used for isoelectrofocusing as described before (Hirai 1982).

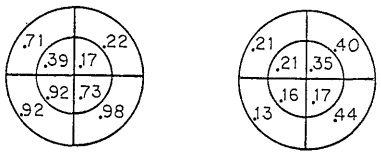

Fig. 2. Distribution of the two types of chloroplasts in the parasexual hybrid calli. Two month old parasexual hybrid calli were sub-divided into eight parts and the chloroplast types in each of them were analyzed by isoelectrofocusing of FIP. The inner areas of the circles represent the upper part of the calli and the outer areas represent she lower part. The numbers shown are the proportions of the *L* kind of *LS* contained in the total *LS* (*L*+*G'*).

3. RESULTS

FIP in the parasexual hybrid callus

Two months after the cell fusion, tens of calli which grow on the hormone free agar medium were obtained. FIP from some of them was analyzed by the isoelectrofocusing (IEF). The small subunit (SS) of FIP is encoded by the nuclear genome (Kawashima and Wildman 1972) and has the isoelectric point which is species specific. As shown in Fig. 1, *L* has two *SS* bands in the isoelectrofocusing gel and *G'* also has two (the basic one is from *N. gossei* and the acidic one is from *N. glauca*). All of the calli selected on the hormone free medium have four bands (two of them are identical to *L* and the other two to *G'*), which proves that all these calli are hybrids between *G'* and *L*.

On the other hand, the large subunit (*LS*) of FIP is encoded by the chloroplast genome (Chan and Wildman 1972). The *LS* bands from *G'* and *L* were separated clearly in the IEF gel. Thus it is possible to distinguish clearly the FIP containing one kind of *LS* from that containing two kinds of *LS*. Fig. 1 shows that all of the two month old calli analyzed have two kinds of *LS*.

FIP in each of the eight sub-divisions of the parasexual hybrid callus.

Two of the two month old calli were cut into eight parts. Each part was subcultured for one more month and used for FIP analysis. Again, two types of *LS* were detected from all of the eight parts. We measured the intensity of the main bands from *G'* and *L* by a densitometor and calculated the ratio of the intensity of *G'* bands to that of *L* bands. The result shows that the ratio of the two types of chloroplasts are entirely different depending on each of the sub-divisions (Fig. 2), which confirms the previous result (Akada and Hirai 1983). Therefore, the mode of chloroplast separation in the new parasexual hybrid callus is very similar to that in the previous one which was obtained by cell fusion between the two established species.

Table 1. *The types of chloroplasts in single cell equivalent calli.*

Chloroplast type	G'	L	$G'+L$
Number of call	14	13	5
L-fixed=42%			

Chloroplast types were analyzed by isoelectrofocusing of FIP isolated from 32 single cell equivalent calli. The calli harboring G' type of chloroplasts, L type of chloroplasts or both type of them are designated as G', L or $G'+L$ respectively. L-fixed indicates half times the proportion of the calli harboring only one parental type of chloroplasts.

FIP in single cells of the somatic hybrid callus

Several small pieces were subcultured for four more months. Because two months is enough for 4 grams of callus to be regenerated from a single cell, it can be assumed that the callus of the same size regenerated after four months of culture has been originated from a single cell. These single cell equivalent calli were used for FIP analysis. The results were summarized in Table 1. It indicates that 84% of the single cell equivalent calli have only one or the other parental type of chloroplasts and 16% have two types of chloroplasts.

Mathematical estimation of the mode of chloroplast separation

One of our interests in this investigation is to study why these two types of chloroplasts separate at this rate. One possible answer is that the number of chloroplasts in one cell is small enough for two types of chloroplasts to be separated "at random" at this rate, which means no biological factors are involved specifically. We estimated the rate of random separation using the following formulae.

We assume. (1) The total chloroplast number (n) in the cells which have just passed the phase of mitosis is invariable. (2) The ratio of the chloroplast G' to L is 1:1 in the original fused cell. (3) The probability of the cells to have k of the G' chloroplasts and $n-k$ of the L chloroplasts is arranged in the matrix $\{P(k)\}$.

$$\{P(k)\}=\{P(0), P(1), P(2) \cdots P(k) \cdots P(n-1), P(n)\}$$
$$(0\leqq k\leqq n,\ 0\leqq P(k)\leqq 1,\ \Sigma P(k)=1)$$

The matrix $\{P_t(k)\}$ where all of the cells are generated through t rounds of cell divisions from the original fused cell is deduced by the next formulae.

$$\{P_0(k)\}=\{P_0(0)=0, P_0(1)=0 \cdots P_0(n/2)=1, P_0(n/2,+1)=0 \cdots P_0(n)=0\}$$
$$\{P_t(k)\}=\{P_0(k)\}\times(1/2n\subset n)^t\times\{A\}^t$$
$$\{A\}=\{\text{Refer to page 442-443}\}$$

$\{A\}=$

	0	1	$k-1$	k
0	$2nCn$	0	0	0
1	:	$2n-2Cn-1\times 2C1$	0	0
:	:	:	0	0
:	:	:	0	0
:	:	:	0	0
m	$2n-2mCn$	$2n-2mCn-1\times 2mC1$	0	0
:	:	:	0	0
$k/2$	:	:	$2n-kCn-(k-1)\times kCk-1$	$2n-kCn-k\times kCk$
:	:	:	:	:
$n/2$	$2n-nCn$	$2n-nCn-1\times C1$	:	:
:	0	0	:	:
$k/2+m$	0	0	$2n-(k+2m)Cn-(k-1)\times 2mCk-1$	$2n-(k+2m)Cn-k\times k+2mCk$
:	0	0	:	:
:	0	0	:	:
:	0	0	:	:
:	0	0	$2n-(k+2)Cn-(k-1)\times k+n-2Ck-1$	:
$k/2+n/2$	0	0		$2n-(k+n)Cn-k\times k+nCk$
:	0	0	0	0
:	0	0	0	0
n	0	0	0	0

(n=even numbers, $0\leqq k<n$, $0\leqq m(n/2)$

Using these formulae, we calculated for $n=6$, 10, 20 and $t=20$, 30, 40, 50, 60. The results about $P(0)$ are shown in Table 2. $P(0)$ is the probability of the cells to have no G' type of chloroplast but L type of chloroplasts. From the experimental data concerning with the single cell equivalent calli, half times the proportion of the calli which have only one parental type of chloroplasts (L-fixed) was 42%, which corresponds to the $P(0)$ for $n=10$ and $t=40$.

4. DISCUSSION

In our attempt to improve the system to investigate the mode of separation of chloroplasts, we utilized the B_1F_1 of *N. gossei* and *N. glauca*; G'. In this plant, the chloroplasts are maternally inherited from *N. gossei* and the nuclear chromosomes are composed of those from *N. glauca* and *N. gossei*.

The genetic tumor produced on the hybrid between *N. glauca* and *N. langsdorffii* are considered to be caused by complementation of gene(s) on a single *N. langsdorffii* chromosome fragment and a large number of genes distributed over many chromosomes from *N. glauca* (Ahuja 1968). The callus induced from the hybrid can grow on the hormone free medium. This hormone independency is useful for selecting the parasexual hybrid between *N. glauca* and *N. langsdorffli* (Carlson 1972).

In this paper we described that this selective marker works for selecting the somatic hybrid callus between G' and *N. langsdroffli* as well as between

$k+1$	$n-1$	n
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
$2n-(k+2(Cn-(k+1)\times 2Ck+1$	0	0
⋮	$nC1\times nCn-1$	nCn
⋮	⋮	⋮
$2n-(k+2m)Cn-(k+1)\times k+2mCk+1$	⋮	⋮
⋮	⋮	⋮
⋮	$n-2mC1\times n+2mCn-1$	$n+2mCn$
⋮	⋮	⋮
⋮	⋮	⋮
$2n-(k+n)Cn-(k+1)\times k+nDk+1$	⋮	⋮
0	⋮	
0	$2C1\times 2n-2n-1$	⋮
0	0	$2nCn$

N. glauca and *N. langsdroffli*. The intensity of the SS bands of the FIP from G' was measured by a densitometor. The ratio of the SS band inherited from *N. glauca* to that inherited from *N. gossei* was almost 6:4. This suggests that about 60% of the nuclear genes in G' is inherited from *N. glauca* and is enough to express the hormone independency by the complementation with the gene(s) from *N. langsdroffli*. We also attempted to make a somatic hybrid between the F_1 (*N. gossei*×*N. glauca*) and *N. langsdoriffli*, but it was not successful. In the F_1 the ratio of the SS band inherited from *N. glauca* to that from *N. gossei* was 4:6.

We assumed that the two types of chloroplasts separate at random. On the basis of this assumption we estimated the rate of separation mathematically. Our experimental data (Table 1) are in agreement with this estimation (Table 2) if the total chloroplast number in one cell is 10 immediately after cell divisions (the number should be 20 before cell divisions) and the two month old callus is composed of the cells which have passed 40 times of cell divisions. There is a report describing that the chloroplast number in one cell varies between 9 and 16 in the suspension culture cells of *N. tabacum* (Thomas and Rose 1983). The number of cell divisions can be estimated by comparing the diameter of a protoplast with that of callus; this is approximately 30 in the two month old callus, and those cells which is dividing vigorously should have passed nearly 40 times of cell divisions. All these available informations support the assumption that the two types of chloro-

Table 2. *Estimation of separation rate of the two types of chloroplasts in the parasexual hybrid callus between G′ and N. langsdorffii.*

n \ t	20	30	40	50	60
6	41	46	49	49	50
10	28	37	42	46	47
20	10	18	26	31	35

The probabilities of the cells which have only L type of chloroplasts $\{P(0)\}$ were calculated according to the formulae described in the results and expressed by percent.

plasts separate at random in a parasexual hybrid callus between *G′* and *N. langsdorffii.*

It is reported that in the state of regenerated plants more than 90% of a parasexual hybrid have only one or the other parental type of chloroplasts (Chen *et al.*). This may also be explained by the random chloroplast separation, considering that in a callus, a rudiment of a plantlet is developed from a nodule where cells are dividing vigorously and that in a plant only the cells in apical meristems are dividing. Therefore, cells in a plant must have passed much more times of cell divisions than those in a two month old callus, so that the random chloroplast separation should have proceeded further in a plant than in a two month old callus as shown in Table 2.

We wish to thank Dr. Shain-dow Kung, Ms. Jennifer Stengle and Ms. Debra Katz for critical reading the manuscript and Mr. Wei-hsiung Shen for calculating the formulae. This research was partly supported by a Grant-in-aid for Scientific Research from The Ministry of Education, Science and Culture, Japan.

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