PHYSIOLOGICAL AND BIOCHEMICAL STUDIES ON POLLEN EMBRYOGENESIS OF *NICOTIANA* SPECIES

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General Introduction

Embryogenesis of pollen grains was first observed in aseptically cultured anthers of Datura innoxia (Guha and Maheshwari 1964, 1966). Their observations demonstrated totipotency in plant cells and laid the foundation for the haploid breeding method. If it is possible to produce many haploid plants from pollen grains and utilize haploidy traits, theoretically we can obtain homozygous pure line with desired phenotypes in a shorter period than required for previous breeding methods (Jensen 1977).

Pollen embryogenesis has been observed in ca. 200 plant species (Maheshwari et al. 1982) and the haploid breeding method was first successfully employed with tobacco (Nakamura et al. 1975). In most other crops, however, the frequency of successfully inducing pollen embryogenesis was too low for practical use. Therefore it is important to study the induction mechanism of tobacco pollen embryogenesis as a possible model system for other species. Such knowledge should contribute to both a better general understanding of totipotency and aid in developing haploid breeding methods for more crops.

In this research, we established a culture method that utilizes a highly homogeneous population of pollen grains. This method allows us to induce pollen embryogenesis at a frequency high enough to study the biochemi-

cal events taking place in the pollen grains during the early period of pollen embryogenesis. Secondly, we attempted to find out any substantial changes which seemed to be related with the induction of pollen embryogenesis by applying the experimental system we have just worked out. In fact, we found out some meaningful changes in protein phosphorylation which coincide with pollen dedifferentiation.

Chapter I

Studies on Conditions for Cell Division and Embryogenesis in Isolated Pollen Culture of *Nicotiana rustica*.

Abstract

A method for the induction of a high rate of cell division and embryogenesis of *Nicotiana rustica* pollen was developed. Binucleate pollen grains were fractionated by Percoll density gradient (35/45%) centrifugation and cultured in 0.4 M mannitol at 30°C (the 1st culture). After 3 days in culture pollen was recollected by a 2nd Percoll fractionation (0/30%) and transferred to and cultured in a medium containing the Murashige-Skoog (MS) macro-elements, 0.4 M mannitol, 40 mM galactose, 3 mM glutamine, and 5 µM ABA for 10 days (the 2nd culture). The cell population consisting of about 80% dividing pollen was transferred to a MS medium containing 0.4 M mannitol, 3 mM glutamine, and no phytohormone (the 3rd culture), where about 40% of dividing pollen developed into embryos or embryogenic calli.

Introduction

Numerous studies have demonstrated in vitro embryogenesis either through anther culture or pollen culture (Maheshwari et al. 1982). However, the induction mechanism of pollen embryogenesis is entirely unknown. The method most widely used has been anther culture, where a majority of pollen grains loses their viability and a limited number shows embryogenic response. It is also possible to induce embryos from the isolated pollen (Nitsch 1974, Rashid and Reinert 1980, Tyagi et al. 1979, Weatherhead and Henshow 1979, Wernicke et al. 1978). In these cases the preculture of anthers or cold treatment of flower buds (Rashid and Reinert 1980) prior to isolation of pollen grains has generally been required. Thus, previously reported culture methods have not been suitable for precise study of the initial stages of pollen embryogenesis.

Recently, Imamura and his coworkers(1982) and Ghandimathi (1982) were successful in the induction of embryos from isolated pollen of Nicotiana plants without any pretreatment of the anthers or buds. Immature pollen grains were cultured in water for a few days, after which basic nutrients was supplied. The frequency of embryogenesis in these isolated pollen cultures was similar to those reported by other methods. Nevertheless, this method seemed suitable for study of the early events responsible for pollen embryogenesis. Therefore, we have attempted to establish a method whereby high

rates of cell division and embryogenesis from *Nicotiana rustica* pollen were possible.

Materials and Methods

Plant Materials. Plants of *Nicotiana rustica* (seeds were supplied by Iwata experimental station, Japan Tobacco Co.) were grown in a greenhouse, but seasonal differences in the successful pollen culture were experienced. Data presented in this paper were obtained from plants grown mainly in winter (November to February) when the most reproducible results were obtained.

General Procedure for Preparation and Culture of Isolated Pollen. The following was a standard procedure established by the present investigation. Details of the manipulation will be described in each experiment.

i) Isolation of Pollen Grains. Pollen was isolated by a method similar to the one described by Nitsch (1974). Flower buds whose corolla length was 6-9 mm were collected. Anthers were excised from buds and immersed in 8 % sodium hypochlorite for 10 min, then washed with sterilized-distilled water. Pollen grains were isolated from anthers by gently grinding with a pestle in 0.4 M mannitol solution, followed by filtration through nylon mesh (53 µm pore size). Pollen grains were washed twice by centrifugation (150 xg , 1 min). Finally pellets (pollen grains) were suspended in 1 ml of 0.4 M mannitol.

ii) Percoll Fractionation of Pollen Grains. Pollen grains were further fractionated by Percoll discontinuous density gradient centrifugation. A 1 ml suspension of pollen was layered on 1.5 ml each of 35%(top)-

45%(bottom) Percoll solution containing 0.4 M mannitol. Following centrifugation (450 xg , 5 min), pollen grains remaining at the interface of the 35/45% Percoll were collected with a pipette. These were washed then twice with 0.4 M mannitol by centrifugation (150 xg , 1 min).

iii) Culture Methods.

The 1st Culture : Pollen grains obtained by the Percoll fractionation were suspended in 0.4 M mannitol at $3x10^4$grains/ml and cultured in a petri dish for 3 days. Unless otherwise mentioned each culture was adjusted to the cell density indicated above and placed at 30°C in the dark.

The 2nd Culture : After the 1st culture, pollen was recollected by centrifugation(150 xg , 1 min) and suspended in 0.4 M mannitol containing Murashige-Skoog(MS) macro-elements (Murashige and Skoog 1962), 40 mM galactose and 3 mM glutamine. pH of the medium was adjusted to 6.8, as recommended by Rashid and Reinert(1983). A 2 ml of the pollen suspension was pipetted into a petri dish (3.5 cm in diameter). In some experiments, the 2nd culture was commenced simply by adding nutrients to the 1st culture. No significant differences in the efficiency of pollen divisions were observed by using the alternative method.

The 3rd Culture : Ten days after the beginning of the 2nd culture, cells(pollen grains) were collected and washed by centrifugation (150 xg , 1 min), then resuspe-

nded in MS medium lacking phytohormone but containing 0.4 M mannitol and 3 mM glutamine (pH 5.8).

Pollen Viability. Pollen size and fluorescent dye staining were the criteria employed to discriminate pollen viability. Living pollen was stained with fluorescein diacetate (0.1 mg/ml), while dead shrivelled pollen did not fluoresce under the fluorescent microscope.

Pollen Division. Division of pollen was always associated with the formation of septa (Fig. I-2A). The presence of nuclei in daughter cells was confirmed by staining with 4,6-diamidino-2-phenylindole (5 μg/ml) after ethanol fixation (Fig. I-2B).

Results

Percoll Fractionation. In previous work (Imamura et al. 1982), total pollen was used for in vitro culture, but the frequency of dividing pollen was not sufficiently high for the study of pollen embryogenesis. Thus, Percoll density gradient (0/35/40/45/50/55%) centrifugation was employed in order to collect a uniform pollen population which might result in high cell division frequency. As shown in Table I-1, pollen remaining in interfaces of the 35/40% Percoll and the 40/45% Percoll gave the highest rate of dividing pollen. The fractions rich in viable pollen tended to show higher division capacity with exception of the top fraction (0/35%). Thus, pollen collected from 35/45% Percoll was used for the following experiments.

Mannitol Concentration. Effect of mannitol in the medium on pollen viability and division in the 1st and 2nd culture is shown in Fig. I-3. Inclusion of mannitol favored both pollen viability and division. Optimum concentration was in the range of 0.3 to 0.5 M, and therefore 0.4 M mannitol was selected as the standard osmotic stabilizer in the 1st and 2nd culture.

Period of the 1st Culture. Maximum pollen division was achieved if the 1st culture period was 3 days (Table I-2). When this 1st culture was not done, pollen division was rarely observed and the accumulation of starch grains similar to that of pollen maturation (Fig. I-1E) occurred.

Evaluations of the 2nd Culture Medium. A carbon source such as galactose in the 2nd culture medium was essential for the induction of division (Table I-3). Addition of MS macro-elements enhanced the rate of the pollen division. Preliminary studies on the effect of sugars suggested that sucrose was effective as galactose in inducing pollen division. However, in the medium containing sucrose the dividing pollen tended to form larger embryos or calli, but in the medium containing galactose as a sole carbon source no further development of the divided pollen took place. Upon transferring the divided pollen from the medium containing galactose to the 3rd culture medium, simultaneous embryogenic development was observed (Fig. I-5). Thus for convenience of counting the pollen divisions and synchronization of embryogenesis following the 2nd culture, galactose was used in the 2nd culture.

Several phytohormones were tested at concentrations from 10^{-5} to 10^{-9}M. Only cis-trans abscisic acid (ABA) at 10^{-5} to 10^{-7} M was found to be effective in increasing the percentage of dividing pollen. Other phytohormones such as indoleacetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzyladenine (BA) and gibberellin A_3 (GA_3) were not effective in increasing the percentage of dividing pollen (Fig. I-4), and each of them in concentrations greater than 10^{-5}M substantially depressed pollen viability. Even if galactose was rep-

laced by sucrose, ABA still enhanced pollen division, but suppressed the growth of dividing pollen into embryos or embryogenic calli in the 2nd culture (data not shown).

<u>2nd Percoll Fractionation.</u> Experiments mentioned above were conducted to assess conditions for maximum rates of the pollen division. To achieve further increase in pollen division a 2nd Percoll fractionation (0/25/30/35/40/45%) after the 1st culture was employed. Pollen obtained from the low density Percoll fractions (0/25%, 25/30%) resulted in higher rates of the pollen division (Table I-4).

<u>Effects of ABA and Glutamine on the Embryogenesis.</u> Using the pollen obtained by the 2nd Percoll fractionation the effect of ABA and/or glutamine supplied in the 2nd culture medium on embryo or embryogenic callus formation was studied (Table I-5). Dividing pollen was transferred to the 3rd culture after 10 days in the 2nd culture to observe embryogenic development. Additions of both ABA ($5x10^{-6}M$) and glutamine (3 mM) in the 2nd culture medium stimulated the development of dividing pollen into embryos or embryogenic calli in the 3rd culture (Fig. I-5).

Discussion

Investigations were carried out to establish conditions whereby a high frequency of cell division and embryogenesis of *Nicotiana rustica* pollen would be feasible. The most significant finding in this study was the necessity of a primary culture lacking any nutrients in the medium. We denote this as "the starvation period". During the starvation period some physiological changes responsible for pollen embryogenesis seem to be taking place. Optimum length of the starvation period was determined to be 3 days at 30°C. This culture period corresponds to that reported by Dunwell (1981) for his method, namely, pretreatment of excised anthers in a water-saturated atmosphere at 23°C. Our unpublished results indicate that the temperature of the 1st culture was also critical for the appropriate starvation period. Under low temperature (eg. 15°C), 7 to 9 days were needed as the optimum period for the 1st culture. This finding is in agreement with that reported by Rushid and Reinert (1980, 1983) for the optimum duration of cold treatment of buds, namely, 10 days at 10°C. In previous methods of pollen culture, preculture of anthers or cold treatment of flower buds before the isolation of pollen was generally required. A comparable situation to starvation of pollen may occur under these conditions.

This investigation also pointed out that pollen selected by Percoll fractionation could be used for the induction of division. Percoll fractionation facilitated

the collection of pollen populations of uniform density. This was visibly confined by a similar accumulation of starch grains. Based upon microscopic observation, pollen obtained from the 1st Percoll fractions, 0/35%, 40/45%, and 50/55% were in the early-binucleate stage (large vacuole, no starch grain), mid-binucleate stage (small or no vacuole, a few starch grains), and the late binucleate stage (many and large starch grains), respectively (Fig. I-1 A,B,C). In previous studies of Nicotiana tabacum and some other plants, anthers suitable for the induction of pollen embryogenesis contained uninucleate to early binucleate pollen (Sunderland and Dunwell 1977), whereas pollen grains which showed embryogenic response in the present study were originated from mid-binucleate pollen grains.

In Nicotiana tabacum anther cultures, Horner and Street(1978) speculated that embryos originated from specific pollen, called S(smaller) grains, which existed at low frequency and had predetermined embryogenic capacity in intact anthers. This hypothesis has been supported by Rashid and Reinert, and a few other investigators (Rashid 1983). In our case embryogenic pollen grains could be collected by a 2nd Percoll fractionation performed right after the starvation treatment (the 1st culture). These pollen grains possessing low density microscopically resemble the embryogenic pollen grains obtained from precultured anthers (Sunderland and

Dunwell 1977, Wernicke et al. 1978) or cold-treated buds (Rashid and Reinert 1980). However, these pollen grains did not originate from the so-called S grains but from the mid-binucleate pollen grains collected by the 1st Percoll fractionation. The decrease in the density of pollen was probably due to the digestion of starch grains during the starvation period.

Our procedure requires a 2nd culture following the starvation period for inducing pollen division. The 2nd culture was also important in improving the frequency of pollen division. Studies of factors contained in the 2nd culture medium disclosed that a carbon source, such as galactose, was essential for inducing pollen division, and that MS macro-elements and ABA were effective in stimulating pollen division. Additions of both glutamine and ABA to the 2nd culture medium were beneficial in inducing divided pollen to undergo a high frequency of embryonic development in the 3rd culture.

Table I-1. Percoll fractionation of pollen prior to the 1st culture and the percentage of dividing pollen in respective fractions.

In this case the 2nd culture was commenced simply by adding nutrients to the 1st culture. The dividing pollen was counted 3 weeks after the culture.

Fraction No.	%Percoll	%Pollen[a]	Viability(%)		%Dividing pollen
			I[b]	II[c]	
I	0/35	6	92	84	6(3-10)[d]
II	35/40	10	84	83	34(28-40)
III	40/45	14	76	57	38(32-44)
IV	45/50	21	22	14	7(4-11)
V	50/55	17	12	7	1(0-4)
VI	55/	32	32	5	1(0-4)

a : The percentage of total pollen found in corresponding fractions.

b : At the beginning of the 1st culture.

c : At the end of the 1st culture.

d : Numbers in parentheses are 95% confidence intervals for a binominal distribution.

Table I-2. Relationship between the duration of the 1st culture and the rate of the dividing pollen.

After the various period of the 1st culture (10 ml suspension in a 10 cm petri dish), 1.8 ml each of the suspensions from the culture was transferred to a 3.5 cm petri dish and MS macro-elements, galactose (final 40 mM) and glutamine(final 3 mM) was added. The dividing pollen was counted about 3 weeks after the addition of nutrients.

Duration of the 1st culture (day)	%Dividing pollen
0	1(0-4)[a]
1	19(14-24)
2	28(22-34)
3	48(42-54)
5	20(15-26)
7	16(11-21)

a : Numbers in parentheses are 95% confidence intervals for a binominal distribution.

Table I-3. Effect of the 2nd culture medium on the rate of dividing pollen.

After the 1st culture pollen was collected by centrifugation, washed and resuspended in each medium. Dividing pollen was counted 3 weeks after the culture.

2nd culture medium[a]	% Dividing pollen
Macro-elements	0(0-1)[e]
M[b]	0(0-1)
Macro-elements + glutamine[c]	0(0-1)
Galactose[d]	31(25-37)
Macro-elements + galactose	48(42-54)
Macro-elements + galactose + glutamine	49(43-55)
M + galactose	51(45-57)

a : Each medium contained 0.4 M mannitol and was adjusted to pH 6.8.

b : Contains all components of MS medium except sucrose and phytohormones.

c : Glutamine was added at a concentration of 3 mM.

d : Galactose was added at a concentration of 40 mM.

e : Numbers in parentheses are 95% confidence intervals for a binominal distribution.

Table I-4. The 2nd Percoll fractionation and the frequency of dividing pollen in respective fractions.

The pollen in each fraction was washed by and cultured in the 2nd culture medium in the same manner as described in **Materials and Methods.**

Fraction No.	%Percoll	%Pollen[a]	Viability(%)[b]	%Dividing pollen
1	0/25	12	100	78(72-83)[c]
2	25/30	40	99	66(60-72)
3	30/35	18	93	46(40-52)
4	35/40	7	23	1(0-4)
5	40/45	7	0	0(0-1)
6	45/	16	0	0(0-1)

a : The percentage of total pollen found in corresponding fractions.

b : At the beginning of the 2nd culture.

c : Numbers in parentheses are 95% confidence intervals for a binominal distribution.

Table I-5. Effects of glutamine and ABA in the 2nd culture medium on division and embryo or embryogenic callus formation.

After the 1st culture, the 2nd Percoll fractionation was conducted and the pollen floating on 30% Percoll solution was cultured in the media indicated. After the 2nd culture (10 days), pollen was collected by centrifugation and provided for the 3rd culture. Ten days after the start of the 3rd culture, embryogenic calli larger than 100 μm were counted.

	2nd culture media[a]			
	+None	+glutamine[b]	+ABA[c]	+glutamine, ABA
Dividing pollen in the 2nd culture(%)	52(46-58)[d]	46(40-52)	78(72-83)	78(72-83)
Embryo or embryogenic callus larger than 100 μm(%)[e]	15(10-20)	12(8-17)	6(3-10)	32(26-38)

a : Glutamine was omitted from the one described in **Materials and Methods** for the 2nd culture medium.

b : Glutamine was added at a concentration of 3 mM.

c : ABA was added at a concentration of 5 μM.

d : Numbers in parentheses are 95% confidence intervals for a binominal distribution

e : The rate to total No. of pollen grains existing at the start of the 3rd culture.

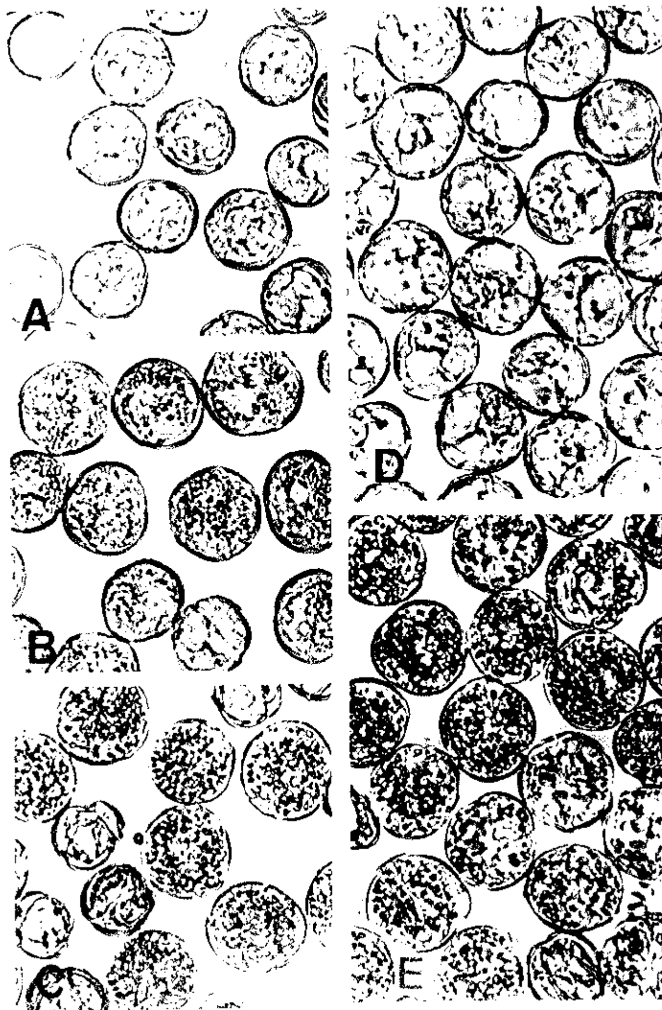

Fig. I-1. Microscopic view of Nicotiana rustica pollen cultured in vitro. A,B,C. Pollen obtained by the Percoll fractions, 0/35%, 40/45%, 50/55% in Table I-1, respectively. D. Pollen grains obtained from the 2nd Percoll fractionation (0/30%). E. Pollen grains sampled from (B) and cultured in the 2nd culture medium for 1 day without the 1st culture. Bar = 50 μm.

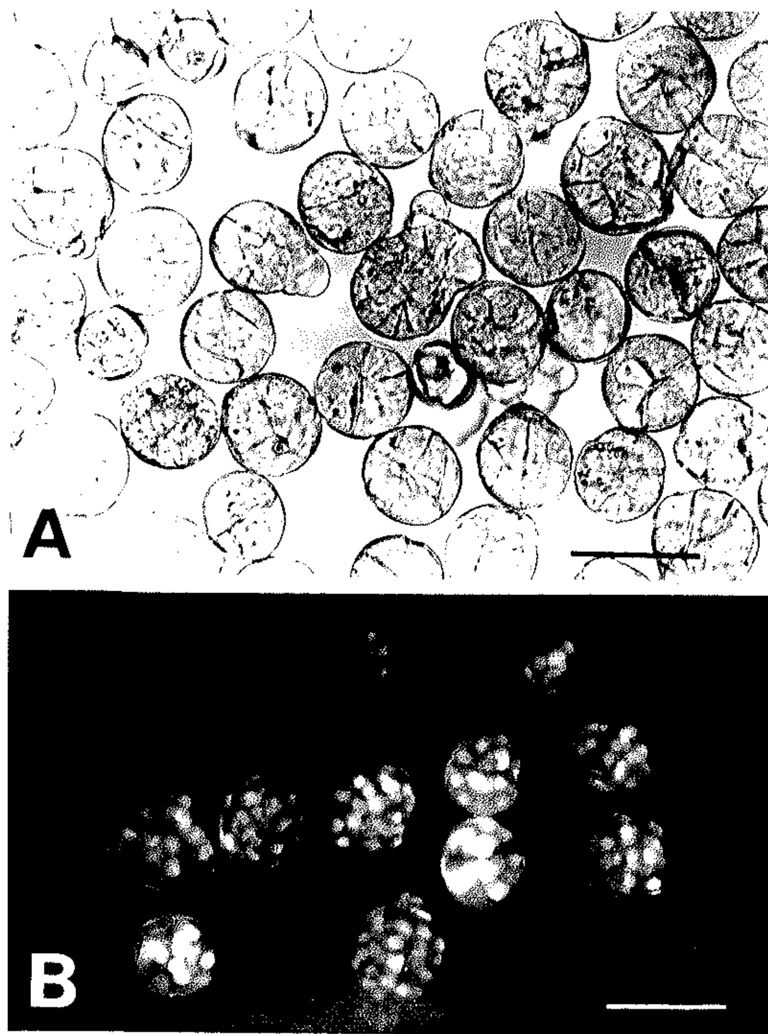

Fig. I-2. Microscopic view of dividing pollen at day 10 in the 2nd culture. Following the 1st culture, the pollen obtained by the 2nd Percoll fractionation (0/30%) was cultured in the medium containing the MS macro-elements, 0.4 M mannitol, 40 mM galactose, 5 μM ABA, and 3mM glutamine (pH 6.8). Dividing pollen(A) was stained by 4,6-diamidino-phenylindole and observed under a fluorescent microscope(B). Note the nuclei strongly stained. Bars = 50 μm.

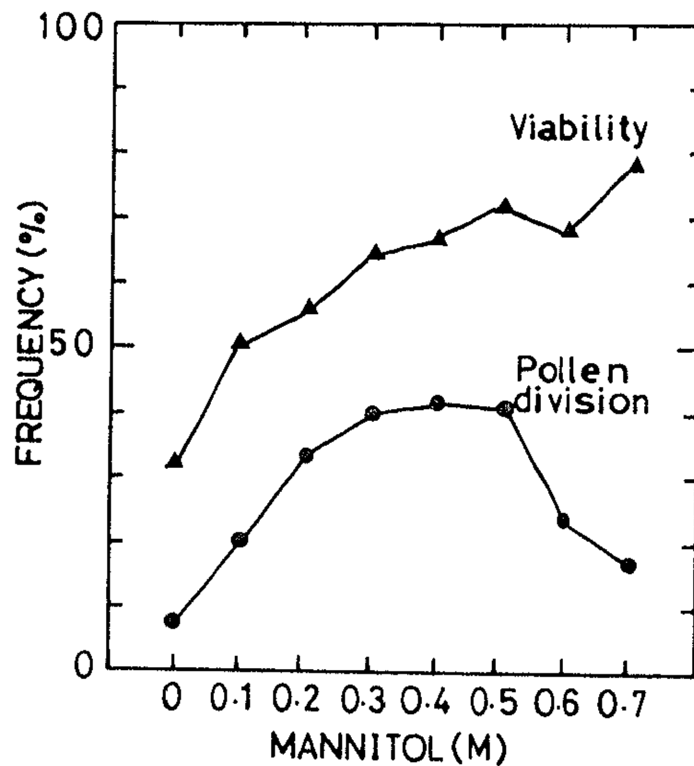

Fig. I-3. Effects of mannitol concentration on pollen viability and cell division. The pollen obtained in the way described in **Materials and Methods** was suspended in mannitol solution at different concentrations of 0 to 0.7 M, and 1.8ml of each suspension was cultured in a petri dish (3.5 cm in diameter). Pollen viability was counted at the end of the 1st culture. Subsequent manipulation and observation of the culture were made as described in Table I-1.

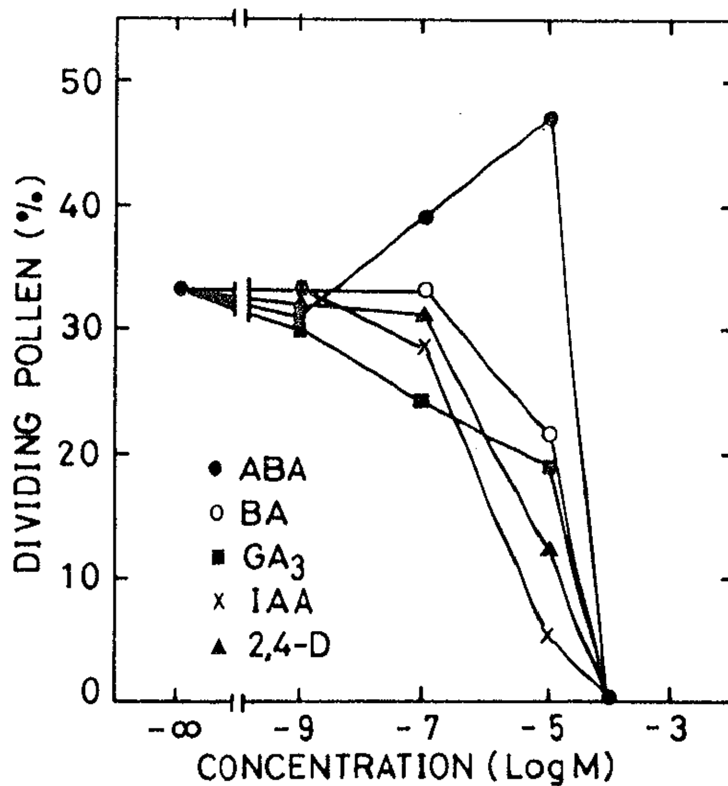

Fig. I-4. Effects of phytohormone in the 2nd culture medium. The method of culture and observation were same as those described in Table I-3. The basal medium was same as the one described in **Materials and Methods.**

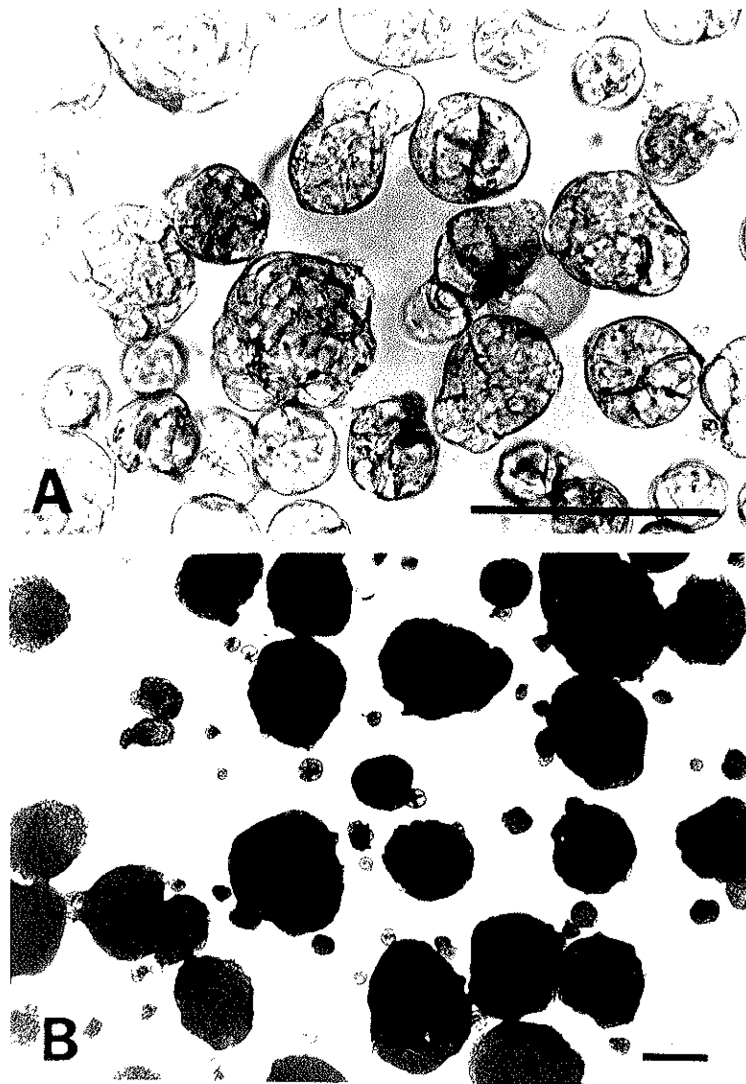

Fig. I-5 Pollen embryogenesis in the 2nd(A) and 10th(B) day of the 3rd culture. Following the 2nd culture, the dividing pollen (Fig. 2 A) was transferred to MS medium (pH 5.8) containing 0.4 M mannitol, 3 mM glutamine and no phytohormone. Bars = 100 μm.

Chapter II

Control of Developmental Pathway of Tobacco Pollen in vitro.

Abstract

We developed a new method for culture of isolated pollen. Using highly homogeneous populations of immature pollen grains of Nicotiana tabacum L. prepared by applying Percoll density gradient centrifugation, we could direct their developmental pathway by regulating certain culture conditions. When the pollen population was cultured in basal medium with glutamine, most pollen grains underwent normal maturation. On the other hand, when first cultured in basal medium without glutamine, most pollen grains did not mature but after transfer to medium with glutamine and sucrose began to divide. This method for inducing pollen cell division was possible only with mid-binucleate pollen grains which are characterized by having no central vacuole and no or only a few starch grains. Evidently, some essential changes responsible for the embryogenic response can be induced by glutamine starvation only in pollen grains at a specific stage.

Introduction

Since the first observation of embryogenesis in pollen by Guha and Maheshwari (1964, 1966), this phenomenon has been an important object of physiological studies in the field of plant cell differentiation and breeding. Androgenesis has been reported in many plant species but its application to practical breeding work was limited mainly because of its low frequency. Increased understanding of the induction of embryogenesis by pollen should help increasing the frequency of the embryogenic response in vitro and its application to breeding work. In previous studies it had been necessary, prior to the isolation of pollen from anthers, to subject the flower buds to a cold-treatment (Rashid and Reinert 1983) or to preculture the anthers on solidified media (Nitsch 1974 a; Wernicke et al. 1980) in order to secure an embryogenic response at high frequency. However, since the essential events responsible for the induction of pollen embryogenesis take place inside of anther wall during these pre-treatment periods, the critical physiological and biochemical events responsible for this process could not be studied.

Recently, Imamura et al.(1982) and Ghandimathi (1982) reported that tobacco pollen grains isolated from fresh anthers without any pre-treatment showed embryogenic response if they were first cultured in plain water for several days and then transferred to a

standard medium. We developed their method further using _Nicotiana rustica_, and showed that high frequencies of pollen embryogenesis could be induced using immature pollen grains at the mid-binucleate stage (Kyo and Harada 1985). However, it was difficult to attain in vitro maturation and germination capacity of the immature pollen grains of _N. rustica_. Therefore, we could not clarify the main factor(s) determining the developmental pathway of immature pollen from gametophytic to sporophytic. Using _N. tabacum_ we now show that mid-binucleate pollen grains can be directed to either the gametophytic or the sporophytic pathway by regulating their culture condition, particularly the supply of glutamine.

Material and methods

Plant material. Flower buds were collected just before use from *Nicotiana tabacum* L. cv. Samsun plants (seeds from Japan Tobacco Co., Iwata Experimental Station) grown under natural light condition in a greenhouse.

Medium preparation. The medium B (TableII-1) without KH_2PO_4 was first prepared and sterilized by autoclaving (120°C, 15 min). A KH_2PO_4-K_2HPO_4 solution (1 M, pH 7.0) sterilized by filtration was added to the cooled medium to make a final concentration of 1 mM. The pH of the medium was approx. 6.8 as recommended by Rashid and Reinert (1983). Medium A (Table II-1) was prepared by adding 0.1 M glutamine to medium B, and medium C (Table II-1) by adding 0.1 M glutamine and 0.3 M sucrose to medium B. The solutions of sucrose and glutamine were sterilized by filtration. Medium D was prepared just before use, the pH being adjusted to 6.8 by KOH and the medium sterilized by filtration.

Isolation of pollen grains. Anthers were excised from flower buds and immersed in 8 % NaOCl solution for 10 min, then rinsed several times using 50 ml sterile water each time. Pollen grains were isolated by gently homogenizing the anthers with a pestle in medium A, followed by filtration through a nylon mesh (53 µm pore size). The pollen grains were rinsed with medium A by centrifugation (150 xg, 2 x 1 min) and suspended in 1 ml of medium A.

Percoll density gradient centrifugation. Sterile 50, 60, 70% Percoll solutions containing medium A components were prepared by mixing absolute Percoll (from Pharmacia, Uppsala, Sweden), distilled water and concentrated medium (3.3 times concentrated medium C ; 1 M mannitol with 66 mM KCl, 3.3 mM $MgSO_4$, 3.3 mM $CaCl_2$, 3.3 mM KH_2PO_4 and 9.9 mM glutamine, pH 6.8). Absolute Percoll and distilled water were sterilized by autoclaving, and the concentrated medium by filtration. The pollen suspension was layered on the top of a discontinuous Percoll gradient (50-60-70%, 1.5 ml each) in a 10-ml glass centrifuge tube with a screw cap. Following centrifugation (450 xg, 5 min), each pollen population forming bands or a pellet was collected with a pipette and diluted with an equal volume of medium A. To obtain a pollen fraction as homogeneous as possible, the two-step Percoll density gradient centrifugation was repeated and the pollen grains which remained in the interface were collected in the same manner as described above.

Pollen culture. Two aliquots of the pollen suspension were separately rinsed three times with medium A or B, by centrifugation (150 xg, 1 min) and the pollen grains were cultured in each medium at a density of $2x10^4$ grains/ml in 3.5-cm (diameter) Petri dishes (2 ml medium/dish) at 27°C in the dark for 2 d. After this first culture the pollen grains were collected by centrifugation (150 xg, 1 min), rinsed twice with medium

C or D, and cultured as in the first culture.

Pollen viability. Pollen size and fluorescent dye staining were the criteria employed to discriminate pollen viability. Living pollen grains were stained with fluorescein diacetate (0.1 mg/ml), while dead, shriveled pollen grains did not fluoresce under the fluorescence microscope.

Pollen germination. Six hours after the beginning of the second culture, the number of pollen grains with a growing tube were counted under an inverted microscope. More than 300 pollen grains were examined for each run.

Pollen cell division. After 10 d culture, pollen grains were collected, suspended in 80% ethanol, and rinsed once with water, then resuspended in 4',6-diamidino-2-phenylindole (5 $\mu g/ml$). The number of grains with four and more nuclei were counted under a fluorescence microscope, then a slide glass with a dent was used to prevent the pollen grains from drifting away when placing a cover glass. More than 300 grains were examined for each test.

All experiments were repeated at least three times, with three to four replications, with similar results.

Results

We applied Percoll density gradient centrifugation to pollen suspensions obtained from selected flower buds with different corolla length (Table II-2), choosing, for reasons described in the **Discussion**, pollen populations from the Percoll fractions 0/50%, 50/60% and 60/70% obtained from, in the order named 16±1-, 22±1- and 28±1-mm buds. By applying again a two-step Percoll gradient centrifugation (0-50%, 50-60%, 60-70%) to the three pollen population, we could increase further the homogeneity of the pollen populations. In this way we could obtain three highly homogeneous pollen populations with approx. 100% viability at the early-, mid- and late-binucleate stages. These populations were characterized by consisting of pollen grains with a central vacuole and no starch grains; no central vacuole and no or a few small starch grains; and no central vacuole and relatively large starch grains (Fig. II-1a, d,j). We refer to these three stages as Stage II, III and IV, in the order given above.

Stage III pollen. Soon after being isolated from the anthers, Stage III pollen grains did not germinate in medium D. However, when cultured in medium A, approx. 95% of these grains attained the mature form within 2 d of culture, increasing their diameter and developing more cytoplasm (Fig. II-1g,h). Upon transfer from medium A to medium D, pollen-tube growth was observed in

maximally 55% of the grains while the remaining grains burst and died (Fig. II-1i, Table 3). Upon transfer from medium A to medium C, pollen grains burst and died within 2 d without showing any pollen-tube growth or cell division (Table II-3). A few (approx. 1%) pollen grains did not show any morphological change in the first culture in medium A and did not germinate upon transfer to medium D (indicated by arrow in Fig. II-1i). These pollen grains were similar to the S grains mentioned in the **Discussion** but did not undergo cell division in medium C.

On the other hand, when the Stage III pollen grains were cultured in medium B, they showed degradation of the cytoplasm and starch grains, and approx. 90% of the pollen grains showed a characteristic cytoplasm which remained in the center of the pollen grain (Fig. II-1e). When these starving pollen grains were transferred from medium B to medium C, asynchronous cell divisions were observed in numerous (maximum 74%) pollen grains within 10 d (Fig. II-1f, Table II-3). Pollen maturation was observed in the second culture, but only occasionally and at a very low frequency (<1%), while most of undivided pollen grains did not show any noticeable morphological change and some of them shrivelled within 10 d. Pollen grains cultured in medium B for 2 d did not germinate upon transfer to medium D (Table II-3). If the Stage III pollen grains were cultured in medium C from

the beginning, mature pollen grains were found at a similar frequency as that in medium A but no cell division was observed. Upon transfer from medium C to medium D, the germination frequency was approx. 30%, i.e. lower than that of mature pollen cultured in medium A.

Stage II and Stage IV pollen. So far, we have not been able to induce normal maturation nor embryogenic response with younger pollen grains at Stage II. When Stage II pollen grains were cultured in medium A or B, no marked morphological changes were observed. The central vacuole became slightly larger and the cytoplasm became slightly thinner and clearer (Fig. II-1b). Upon transfer from medium A or B to medium C, these grains died within several days after some accumulation of starch grains but without any cell division (Fig. II-1c). When Stage II pollen grains were cultured in medium C from the beginning, they burst and died within several days, showing some accumulation of starch grains.

With older Stage IV pollen grains we could induce maturation but no embryogenic response. When such pollen grains were cultured in medium A or C and transferred to medium D, their behavior was similar as that of Stage III grains. When Stage IV grains were cultured in medium B, they showed degradation of starch grains and appearance of many vesicles and a reticulate cytoplasm (Fig. II-1h). Upon transfer from medium B to medium C, all of them burst within 1 d without any division or germination (Fig. II-1l).

Discussion

As normal pollen development (maturation) proceeds, after the late-uninucleate stage, in intact anthers, the density of the pollen grains increases continuously because of the increase in volume of their cytoplasm and the quantity of starch grains they contain. When Percoll density gradient centrifugation was applied to pollen suspensions prepared from flower buds with different corolla lengths, the density of the major part of the pollen grains in each fraction increased in proportion to the corolla length. When the pollen grains in each fraction were cultured to induce pollen cell division, first in medium B for 2d and subsequently in medium C for 10 d, the highest frequency of division was obtained with the pollen in the Percoll fraction 50/60 obtained from 22±1-mm buds (approx. 60%). Cell division (approx. 10-35%) was also observed in pollen from other fractions, namely, fraction 50/60 from 19±1- and 25±1-mm buds and fraction 60/70 from 22±1- and 25±1-mm buds. A considerable part of pollen grains in these fractions may be at younger or older stages than Stage III (cell-division-inducible stage). Thus, by using the Percoll fractions 0/50, 50/60 and 60/70 prepared from flower buds with corollas, in the order given, 16±1, 22±1 and 28±1 mm long and using, fairly homogeneous pollen populations prepared from these fractions, we could determine stage specificity of the embryogenic

potential. The divided pollen grains induced by the method described above have not developed bigger than 50 µm in diameter in medium C. Further examination will be required for plantlet formation from them at high frequency. According to our preliminary experiments, it is rather difficult to develop the divided grains to large embryos or plantlets at high frequency in the case of N. tabacum comparing N. rustica (see Kyo and Harada 1985).

We noticed occasional slight changes in the correlation between the pollen stage and corolla length, which were probably the consequence of differences in the physiological conditions of the mother plants, depending upon age, growing season and-or climatic factors. To obtain as homogeneous a pollen population as possible, we sampled one anther from each bud and observed its pollen grains under a microscope to eliminate buds containing pollen grains at undesired stages. In our plants there was no noticeable difference in the state of pollen among the five anthers in a bud.

Our results show that normal pollen maturation and formation of embryogenic pollen from mid-binucleate stage pollen grains could be realized with media A and B, respectively. The absence of glutamine seems to trigger certain physiological changes in the pollen grains which are essential for the acquisition of the embryogenic capability. This fact is interesting in view

of the various instances of starvation effects on the developmental program in many organisms, such as spore-forming bacteria, yeast, slime molds, algae and fungi. When isolated pollen grains were cultured in medium B (starvation period), we observed degradation of cytoplasm and starch grains, but only with pollen grains at stage III did we observe a characteristic cytoplasm remaining in the center of the pollen. This aspect resembles that of cultured protoplasts or somatic cells just ready to undergo the first cell division.

In anther cultures of Nicotiana tabacum, anthers containing pollen grains at the first pollen mitosis to the early-binucleate stage (younger than the ones at stage III) are suitable for inducing androgenesis (see Maheshwari et al. 1980). However, Heberle and Reinert (1977) indicated that most suitable pollen stage (binucleate stage) for isolated pollen culture was older than that for anther culture. Dunwell and Sunderland (1974 a) suggest, from microscopic observations, that the embryogenic induction of pollen began after a short period of normal gametophytic development within the cultured anthers. Therefore, the stage of pollen most suitable for embryogenesis must be an older stage than that at the beginning of the anther culture, and this stage is probably close to Stage III. Dunwell and Sunderland (1974 b) also observed that the gametophytic cytoplasm was digested during the embryogenic induction

period before the beginning of cell division. The similarity in the morphological changes in pollen grains observed in both anther culture and our pollen culture indicates the existence of a period of pollen starvation also in the early stage of the anther culture. Thus, we may relate the processes taking place during an early period of anther culture to those observed in our pollen culture method as follows. When anthers suitable for inducing embryogenesis are excised from mother plant, they contain a certain amount of nutrients which allows the immature pollen grains to follow, for a short period, gametophytic development in the excised anthers. However, the nutrient pool will soon be exhausted, and the pollen grains starve and begin self-digestion of starch, etc. Even though the anthers are cultured on a nutrient medium, the nutrients can not easily penetrate the anther wall with its waxy layers. In fact, Aruga and Nakajima (1985) showed that the sugar content in anthers cultured on a medium with sucrose, decreased during the early period of culture. Within the poorly nourished anthers, a limited number of pollen grains at Stage III may begin to follow the sporophytic pathway, with degradation of the gametophytic cytoplasm. Probably, a quite similar process takes place also during the cold treatment period of excised flower buds (10°C, 10 d) in ab initio pollen culture by Rashid and Reinert (1983). As the anther tissues decay, the nutrients in the medium can gradually reach the starved pollen grains. Thus, an

initial period of pollen starvation and the resupply of necessary nutrients to the pollen may be very important factors for pollen embryogenesis.

Horner and Street (1978) proposed that in *Nicotiana tabacum* anther culture embryos arise from specific pollen grains, so called S (smaller) grains, which exist at a low frequency in intact anthers and are distinguishable from normal gametophytic pollen grains by their smaller size and their lighter staining with acetocarmine, and that the embryogenic capacity of the S grains has been determined already in situ. However, our results show that embryogenic pollen grains arise from the mid-binucleate pollen grains which can be obtained from a major part of pollen population in an anther and possess maturation capacity, and indicate that the embryogenic pathway proposed by Horner and Street is not sole. It seems possible that small number of embryogenic pollen grains can be formed by spontaneous starvation in intact anthers as a result of the competition among a large number of pollen grains for a limited amount of nutrients available. In fact, some workers showed that some cultural and physiological conditions of the mother plants of *N. tabacum* were favorable in increasing the number of sterile pollen grains having embryogenic capacity in the intact anthers and-or the frequency of pollen embryogenesis in the anther or pollen cultures. These are, for example: environmental factors; shorter

photoperiod (8 h rather than 16 h), lower temperature (18°C rather than 24°C)(Heberle-Bors and Reinert 1981), nitrogen starvation (Sunderland 1978, Heberle-Bors 1983), and treatment with growth substances; naphthalene acetic acid (NAA), N,N-dimethylaminosuccinamic acid (Alar 85) (Heberle-Bors 1983). We presume that the factors mentioned above disturb the synthesis and-or translocation of nutrients, and deteriorate the nutrient conditions of mother plants, particularly that of the intact anthers during pollen development. Such a condition may facilitate to induce starved state of pollen grains and make them deviate from gametophytic development. As a result, the number of embryogenic pollen grains in situ (equivalent to the S grains) may be increased.

Table II-1. Composition of the culture media used. Figures= concentration in mM.

Components	Medium			
	A	B	C	D
KCl	20	20	20	0
$MgSO_4$	1	1	1	0
$CaCl_2$	1	1	1	0
KH_2PO_4	1	1	1	0
H_3BO_3	0	0	0	2
Glutamine	3	0	3	0
Sucrose	0	0	1	300
Mannitol	300	300	300	0

Table II-2. Distribution of N. tabacum pollen grains fractionated by Percoll density gradient centrifugat

Percoll fraction	Corolla length (mm)				
	16±1	19±1	22±1	25±1	28
0/50	68[a](94)[b]	7(86)	9(81)	4(80)	3(2
50/60	19(45)	42(95)	65(93)	38(91)	9(5
60/70	11(0)	47(14)	17(65)	43(89)	45(
70/	2(0)	4(0)	9(13)	15(45)	43(

a Figures = percentage of pollen

b Figures in ( ) = pollen viability (%)

Table II-3. Percentage of germinated and divided pollen grains of *N. tabacum* when Stage III pollen grains were cultured applying the medium schemes indicated below.

1st medium	2nd medium	Pollen(%)	
		Germinated[a]	Divided[b]
A	C	0(0-1)[c]	0(0-1)
A	D	55(61-49)	0(0-1)
B	C	0(0-1)	74(80-68)
B	D	0(0-1)	0(0-1)

a Counted 6 h after the transfer to the 2nd medium.

b Counted after 10d of culture in the 2nd medium.

c Figures in ( ) = 95% confidence intervals for a binominal distribution.

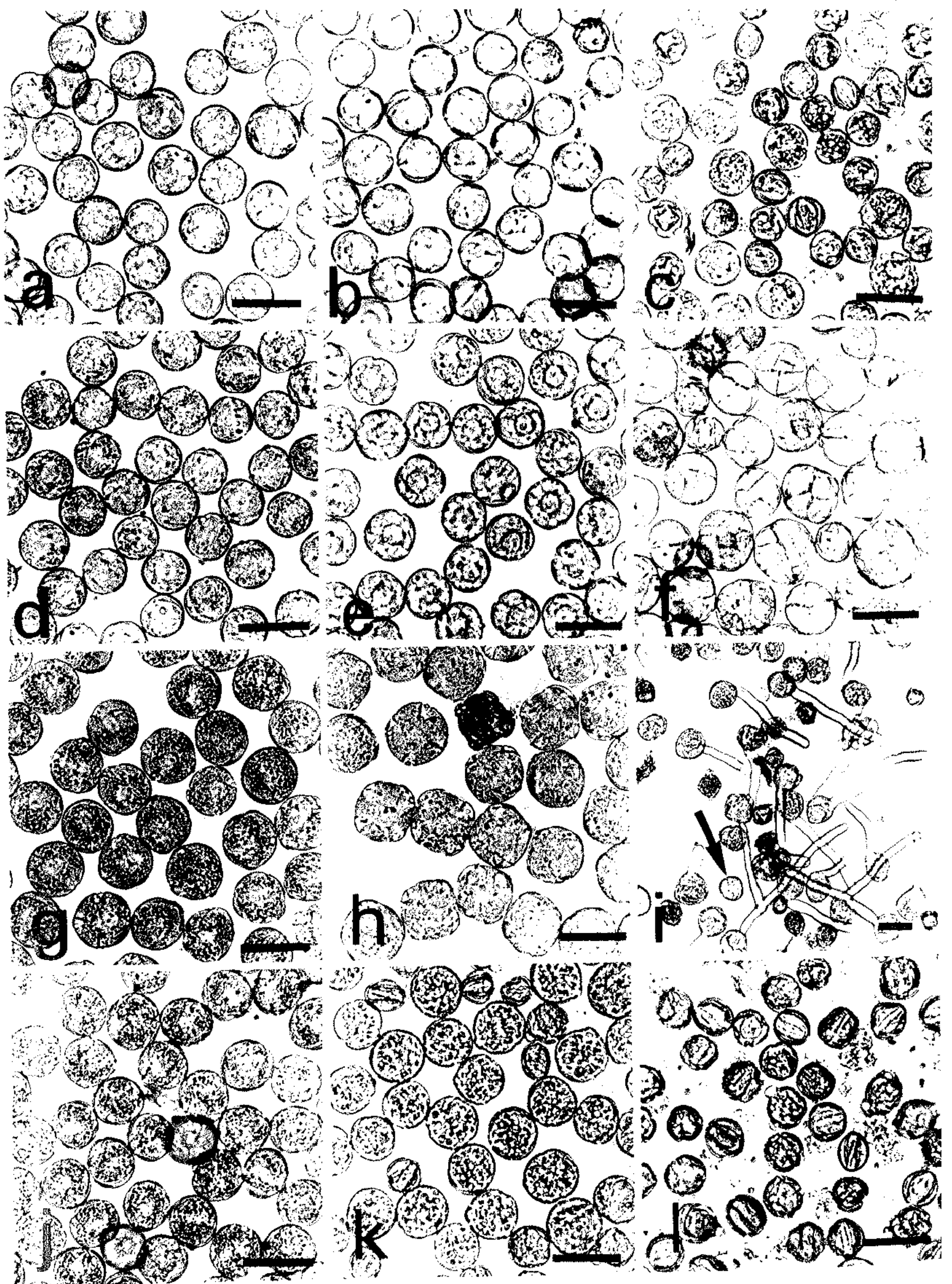

Fig. II-1. Nicotiana tabacum pollen cultured in vitro.

a,d,j Pollen grains at Stages II (a), III (d), and IV

(j) at the start of culture. b,e,k Two days after the start of culture of (a),(d), and (j)in medium B. c,l Two days after transfer of b and k, respectively, to medium C. f Divided pollen 10 d after the start of the second culture in medium C. g,h One and 2 d, respectively, after the start of culture of j in medium A. i Pollen-tube growth 5 h after transfer of mature pollen (h) to medium D. Bars = 50 μm ; magnifications : x260 (a-h, j-l), x130 (i).

Chapter III

Changes in phosphorylated protein pattern of cultured pollen grains during early stage of pollen embryogenesis.

Abstract

We examined the electrophoretic pattern of phosphorylated proteins of the pollen cultured in various conditions. When the mid-binucleate pollen (Stage III) dedifferentiated in basal medium without glutamine, phosphorylation of the proteins **a-d** was clearly detected. When pollen grains matured in medium with glutamine, phosphorylation of proteins **e-i** was evident but it apparently did not occur in proteins **a-d**. Phosphorylation of protein **a-d** was partially or completely suppressed in pollen grains which did not transform to embryogenic cells in medium without glutamine. Some chemical compounds, which promote or inhibit pollen dedifferentiation, changed the electrophoretic pattern of phosphorylated proteins when added to the medium. We conclude that the phosphorylation of proteins **a-d** and that of **e-i** are related with pollen dedifferentiation and maturation, respectively.

Introduction

Embryogenesis of pollen grains has been observed in many plant species but the precise induction mechanism is virtually unknown. This is probably due to the fact that culture methods previously employed for inducing the phenomenon have not been suitable for biochemical analysis.

In **Chapter II,** we reported a new method of *Nicotiana tabacum* pollen culture which allows us to carry out biochemical investigation of pollen embryogenesis (Kyo and Harada 1986). Using a highly homogeneous population of immature pollen grains at a specific developmental stage and regulating certain culture conditions, we could direct the developmental pathway of the pollen grains toward either gametogenesis or embryogenesis. When cultured in basal medium with glutamine, most pollen grains underwent normal gametogenic development, acquiring the ability to form pollen tube, i.e. maturation. On the other hand, when cultured in a medium without glutamine, most pollen grains become embryogenic acquiring the ability to divide, i.e. dedifferentiate. The latter event was observed in only at mid-binucleate stage, but not in younger or older stages. Therefore, some physiological changes responsible for dedifferentiation of pollen may take place in pollen grains at the specific stage by glutamine starvation. Investigation and comparison of the biochemical profiles of cultured pollen grains during maturation and dedifferen-

tiation should reveal reliable biochemical markers associated with the induction of pollen embryogenesis, i.e. dedifferentiation. We considered that such markers should be found in high molecular substances, especially in proteins.

However, we could detect no protein specific to the dedifferentiating pollen grains in 2-dimensional electrophoretic patterns of accumulated or newly synthesized protein, which were visualized by silver staining and fluorography. Recent studies have shown that phosphorylation of certain proteins is an important factor in the expression of various cell functions (Weller 1978, Colowick and Kaplan 1983). In this chapter, we will show that phosphorylation of several specific proteins coincides with the dedifferentiation of pollen grains.

Materials and Methods

Plant Material and Pollen Culture. *Nicotiana tabacum* cv. Samsun plants were grown under natural day-light conditions in a greenhouse. The pollen grains were isolated from 10-12, 15-17, 21-23, 25-27 and 30-35 mm flower buds and fractionated by Percoll density gradient centrifugation as described in **Chapter II**. The pollen grains which remained in the 0/40%, 40/50%, 50/60%, 60/70% and 70/80% Percoll interfaces, were referred to as Fraction I, II, III, IV and V in the order given. Fraction II, III and IV contain Stage II, III and IV pollen grains, respectively, as their major parts (see **Chapter II**). The culturing and transferring of pollen from one medium to another were performed in the manner described in **Chapter II**. Culture media A, B, C and D were prepared according to Table II-1 shown in **Chapter II**. In order to promote the uptake of [^{32}P]-orthophosphate ($^{32}P_i$) by pollen grains, the concentration of KH_2PO_4 in the media A,B and C was decreased to 0 or 0.1 mM. Media with the low KH_2PO_4 concentration of 0 or 0.1 mM are referred to as medium A' or A", B' or B" and C' or C", respectively. Pollen grains were cultured at a density of $2x10^4$ grains/ml in 5-cm Petri dishes (5 ml medium/dish).

Feeding Labeled Compounds to Pollen Grains. Pollen grains were cultured in medium with 10 μCi/ml $^{32}P_i$ (carrier free, Japan Atomic Energy Research Institute) or 10 μCi/ml [^{35}S]-methionine (1000 Ci/mmol, Amersham)

for durations described in the **Results** section.

Sample Preparation and 2-Dimensional Gel Electrophoresis. The pollen grains fed with labeled compounds were collected and rinsed twice with 0.3 M mannitol containing 20 mM KCl by a centrifugation (150xg, 1 min). The pellets (pollen grains) were stored in microcentrifuge tube at -80°C until use. They were homogenized in ice-cold 100 µl 20% trichloroacetic acid (TCA) with a glass-Teflon homogenizer and kept on ice for 1 h. The homogenates were centrifuged (12,000xg, 30 min) and the pellets were rinsed once with ice-cold acetone and then 3 times with ice-cold ether by centrifugation (12,000xg, 15 min). The pellets were dried on ice to remove the ether, then the pellets were resuspended in 20 µl of 9 M urea solution containing 5% 2-mercaptoethanol and 2% Nonidet P-40. After centrifugation (12,000xg, 5 min.), the supernatant was used for micro-scale 2-dimensional gel electrophoresis (Mikawa et al. 1981). The protein contained in the supernatant was separated by nonequilibrium pH gradient electrophoresis (NEPHGE) for the first dimension and 12.5% SDS-polyacrylamide gel for the second. After electrophoresis, the gel was soaked in 10% TCA for 10 min, then in 50% methanol overnight with 3 changes of the methanol. The gel was dried and exposed to X-Omat film (Kodak) for 3-10 days.

Results

In this report the frequencies of germinated and divided pollen in the 2nd culture are indexes of pollen maturation and dedifferentiation in the 1st culture, respectively.

<u>Changes in the frequency of pollen dedifferentiation in relation to the duration of culture.</u> In medium B', the frequency of dedifferentiated pollen grains increased rapidly from 24 h of culture, attained their highest rate within 48 h (Fig. III-1).

<u>Changes in electrophoretic pattern of phosphorylated proteins during the maturation and dedifferentiation.</u> Phosphorylated proteins of pollen in the medium B' or A' were analyzed by 2-dimensional gel electrophoresis and autoradiography (Fig. III-2). In the absence of glutamine, a group of spots **a-d** were intensified in the right half (acidic side) of the pattern after 24 h of culture (**b** consists of 3 or 4 spots), while spot **e** become lighter with time. In the presence of glutamine, spots **a'** and **g-i** appeared denser in the left half (basic side) of the pattern but spots **a-d** scarcely did. The density of the spot **e** did not change with time. Spots **a** and **a'** were in nearly same position but may be different, judging from other results (for example, Fig. III-5 and 10).

<u>Effects of glutamine on the maturation and dedifferentiation of pollen.</u> Fig. III-3 shows the frequency of pollen maturation and dedifferentiation induced in

medium B' with glutamine at various concentrations. When glutamine concentration was high, more pollen grains matured and fewer dedifferentiated. As the concentration of glutamine decreased, the ratio of pollen grains matured to those dedifferentiated was reversed.

Effects of glutamine on protein phosphorylation. As shown in Fig. III-4, the total amount of ^{32}P incorpolated into proteins varied according to glutamine concentration. In the presence of glutamine at a concentration of 0.3 mM or higher, the spots **e-i** were distinctively noted. Their density depended on glutamine concentration. In the absence and presence of glutamine at a concentration of 0.3 mM or below, the spots **a-d** were observed (**d** is not clear in Fig. III-4). Their density was inversely proportional to glutamine concentration.

The pattern change of phosphorylated proteins of cultured pollen at various stages. It was examined whether if the spots **a-d** appear in the case of the pollen at various developmental stages cultured in medium B" for variuos periods (Fig. III-5). The spots **a** and **c** were found as faint spots with Fraction I, and as dense spots with Fraction II after 24 h of culture, while the spots **b** and **d** were not detected with the both fractions within 78 h of culture. In Fractions I and II, no pollen dedifferentiated. With Fraction III, the spots **a-d** appeared most densely and 72% pollen dedifferen-

tiated. With Fraction IV, they appeared considerably and 20% pollen dedifferentiated. With Fraction V, they were not detectable but the spots g-i were densely appeared, and no pollen dedifferentiated. The spot a' indicates different protein(s) from a, judging from other results (Figs. III-4 and 11).

Effects of phytohormone on pollen dedifferentiation. The influence of phytohormones including 6-benzyladenine (BA), 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic Acid (2,4-D), gibberellin A_3 (GA_3) and cis-trans abscisic acid (ABA) on the pollen dedifferentiation was investigated. Unlike other hormones, increasing concentration of BA was inhibitory to the pollen dedifferentiation (Fig. III-6).

Effects of EDTA on the pollen dedifferentiation. The effects of chelating reagents on pollen embryogenesis were previously observed in tobacco anther culture (Matsubayashi and Misoo 1977, Heberle-Bors 1980). Also in our culture system, it was found that EDTA was effective on pollen dedifferntiation at higher concentration above 0.1 mM (Fig. III-7). The effect of EDTA was more evident with Fraction IV than Fraction III. When cultured in medium B" with and without EDTA for 48 h, the pollen grains of Fraction IV showed clear morphological differences between the two treatments (Fig. III-8). In the presence of EDTA, the degenerative cytoplasm remained in the center of the pollen grain, but reticulated cytoplasm was observed in the absence of EDTA. The

former aspect obtained with EDTA resembled to that of dedifferentiated pollen grains with Fraction III (see Fig. II-1e in **Chapter II**).

Effects of BA and EDTA on protein phosphorylation. Fig. III-9 shows the electrophoretic patterns obtained from Fraction III. In the presence of BA (1 μM), the spots **a-f** specifically faded, while in the presence of EDTA (1 mM), most spots other than **a-d** faded. With Fraction IV, as shown in Fig. III-10, in the presence of BA (1 μM), the spots **a-d** were faint, while in the presence of EDTA (1 mM), the spots **a-d** become denser, especially, spots **b** and **c** were remarkable (although the **d** was the least clear). In these experiments, part of the pollen suspension was put aside before the labelling and cultured for the examination of frequency of dedifferentiated pollen grains. The frequencies of control, EDTA-treated and BA-treated lots were 10, 65 and 0%, respectively.

The patterns of phosphorylated protein after the beginning of pollen cell division. It was examined whether if the spots **a-d** appeared or not during the 2nd culture. As shown in Fig. III-11, the spots **a-d** were clearly observed until the 1st day of the 2nd culture. But they became faint on the 4th day and disappeared thereafter.

Protein synthesis during the maturation and dedifferentiation. Changes in total protein content and incorporation of [^{35}S]-methionine into acid insoluble fraction of pollen grains cultured in medium A or B are summa-

rized in Fig. III-12. In the presence of glutamine (in medium A), both the protein content and the incorporation of [^{35}S]-methionine increased in the first 24 h of culture but decreased slightly after 48 h. Without glutamine (in medium B), both decreased gradually over 48 h. Changes in the electrophoretic pattern of newly synthesized proteins in pollen grains during the dedifferentiation and maturation were shown in Fig. III-13. Although the pattern changed with time in both processes, we could not find any protein spots characteristic to the dedifferntiating pollen (the details were not shown in Fig. III-13). Two dimensional electrophoreogram of accumulated proteins which made visible by silver staining, also showed no qualitative differences between the 2 processes (data not shown).

Discussion

Between mature and dedifferentiated pollen induced in vitro, functional and morphological differences were distinctly observed. The former possesses developed cytoplasm and germination ability. The latter shows characteristic cytoplasm which is degenerated and remained in the center, and possesses cell division ability. Therefore, certain substantial differences responsible for such properties must occur between pollen maturation and dedifferentiation process. We expected that high molecular substances, especially proteins, should be qualified for such substances. As for total protein content and rate of protein synthesis, qualitative difference was distinctly noted. During maturation, both of them tended to increase while during dedifferentiation, both of them decreased. Similar results were, in part, previously reported (Bhojwani et al. 1973). Furthermore, as shown in Fig. III-12, the cytoplasm of pollen grains with cell division ability was sparser than that without it. We suppose that degradation of proteins and suppression of protein synthesis are necessary conditions for pollen dedifferentiation. However, in spite of many experiments and careful observations, we could not detect any protein spots specific to dedifferentiated pollen in 2-dimensional gel electrophoretic pattern of newly synthesized or accumulated proteins. We still expect the presence of certain proteinic changes which characte-

rizes the function of dedifferentiated pollen.

Recently, many biochemists and physiologists have been paying attention to participation of protein phosphorylation in regulating cell function responding to environmental change in eukaryotes (for example, Celenza and Carlson 1986, Simanis and Nurse 1986). In our pollen culture system, the environmental change, i.e. glutamine starvation, causes pollen grains to deviate from normal developmental pathway and dedifferentiate. The association of protein phosphorylation with pollen dedifferentiation seemed to be possible. Therefore, we analyzed the phosphorylated proteins of cultured pollen grains by 2-dimensional gel electrophoresis and autoradiography. And we found critical differences between pollen maturation and dedifferntiation. When pollen grains were cultured with glutamine at lower concentrations which allow pollen grains to dedifferentiate, the proteins **a-d** were highly phosphorylated. On the other hand, when cultured with glutamine at higher concentrations which allows pollen to mature, the phosphorylation degree of protein **a-d** was very low and that of other proteins **e-i** was high. Both the intensity of phosphorylation of protein **a-d** and the frequency of dedifferentiated pollen grains coincidently increase after 24 h of culture without glutamine. It is possible, then, that the phosphorylation of proteins **a-d** and **e-i** is associated with pollen dedifferentiation and maturation, respectively.

When pollen grains at various developmental stages were cultured without glutamine, phosphorylation of all proteins **a-d** was found only with pollen populations containing dedifferentiated pollen grains (Fraction III and IV) but partially or scarcely with the other populations (Fraction I, II, and V). Fraction IV was prepared from Stage IV pollen rich population but was considerably contaminated by Stage III pollen grains which possess the potentiality of dedifferentiation (see Chapter II). The intensity of spots a-d was in proportion to the frequency of dedifferentiated pollen in the pollen population. These results add further evidence for the significance of phosphorylation of all proteins **a-d** in respect to pollen dedifferentiation. With Fraction V, a large number of pollen grains matured even in the medium without glutamine within 24 h (data not shown). These pollen grains seemed to contain enough amount of nutrients for maturation so that they could mature without exogenous glutamine. Then the electrophoretic pattern of phosphorylated protein was similar to that obtained from mature pollen grains induced with Fraction III in the medium containing glutamine. This suggest that phosphorylation of proteins **a'** and **e-i** (especially, **g-i**) is associated with the pollen maturation.

To ascertain the association of phosphorylation of proteins **a-d** with pollen dedifferentiation, we examined the electrophoreogram of phosphorylated proteins if

pollen dedifferentiation was inhibited and promoted by supplying some chemicals. Among many chemical substances investigated, we found that EDTA and EGTA, chelators of divalent cation, promote pollen dedifferentiation and that BA inhibits it (data not shown with EGTA). Previously, the effect of chelating reagents on pollen embryogenesis was reported in tobacco anther culture (Matsubayashi and Misoo 1977, Heberle-Bors 1980). The effects of these reagents both on pollen dedifferentiation (Fig. III-6 and 7) and protein phosphorylation pattern (Fig. III-9 and 10) suggest that phosphorylation of proteins a-d is required for pollen dedifferentiation but that of most of other proteins is not. In parallel with the promotion of pollen dedifferentiation by EDTA in Fraction IV (Fig. III-8), the pollen grains showed sparse cytoplasm which is characteristic to dedifferentiated pollen and is clearly different from the reticulated one observed in the absence of EDTA. This result adds further evidence for the significance of the characteristic morphological change in respect to the pollen dedifferentiation and suggests the association of the phosphorylation of protein a-d with the morphological change.

In the previous reports (Matsubayashi and Misoo 1977, Heberle-Bors 1980) and also in our present experiments, EDTA was effective on induction of pollen embryogenesis at the lower concentration (0.1 mM) than the

concentration of divalent cation in the medium (1 mM Ca^{2+} and 1 mM Mg^{2+}). This may suggest that the promotion of pollen dedifferentiation by EDTA is due to side effects other than chelating cations. It was, however, reported that the intracellular calcium ion concentration was submicromolar level in plant cells (Heplar and Wayne 1985). Therefore, it seems to be possible that a very small amount of EDTA dissociated from calcium or magnesium ion enter into the pollen cytoplasm and affect the intracellular calcium ion level. We showed the inhibitory effect of BA on pollen dedifferentiation. It was reported that cytokinin increased the intracellular calcium ion level and, in result, promoted bud formation in protonema of the moss, Funaria hygrometica (Saunders and Hepler 1981, 1982, 1983). The promotive effect of EDTA and inhibitory effect of BA on pollen dedifferentiation may be mediated by a decrease and increase in intracellular calcium ion concentration, respectively. Considering the results described above, we speculate that the intracellular calcium ion concentration have influence on phosphorylation of certain proteins (a-i) and synthesis and degradation of mass protein which should be important factors of the expression of pollen functions, germination and embryogenesis.

In the 2nd culture, pollen began to divide asynchronously after a lag period of several days (data not shown). The phosphorylation of proteins **a-d** took place in an early period of the 2nd culture, but not after

active cell divisions started. This indicates that the phosphorylation of the proteins **a-d** is not related with cell division. The phosphorylation of certain proteins seems to have important significance in the change of the developmental program from gametogenesis to embryogenesis before the beginning of embryogenic cell division, in other words, in the initial process of the expression of totipotency.

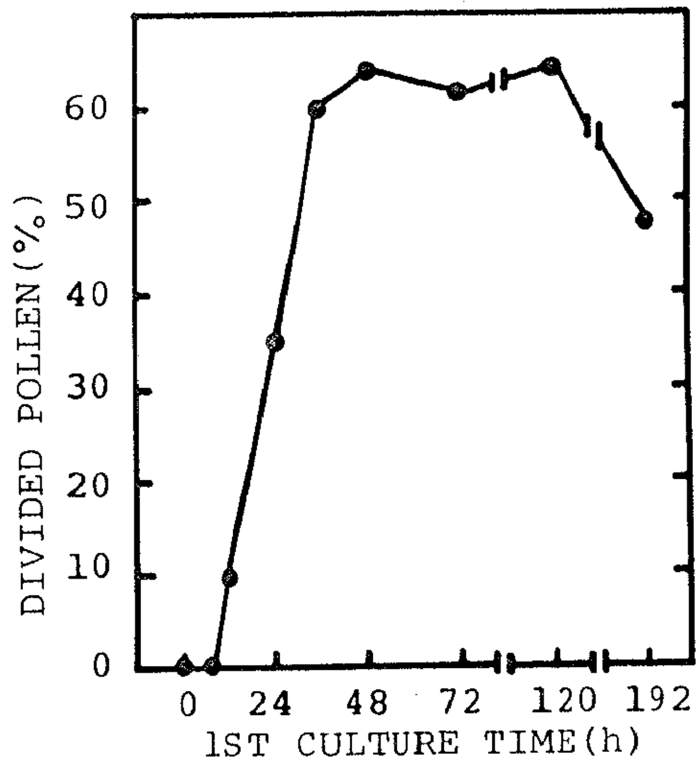

Fig. III-1. Changes in the frequency of dedifferentiated pollen in relation with the duration of culture. Stage III pollen was cultured in medium B' for various periods and then transferred to medium C. The cell division was observed in the same manner as that described in Chapter II.

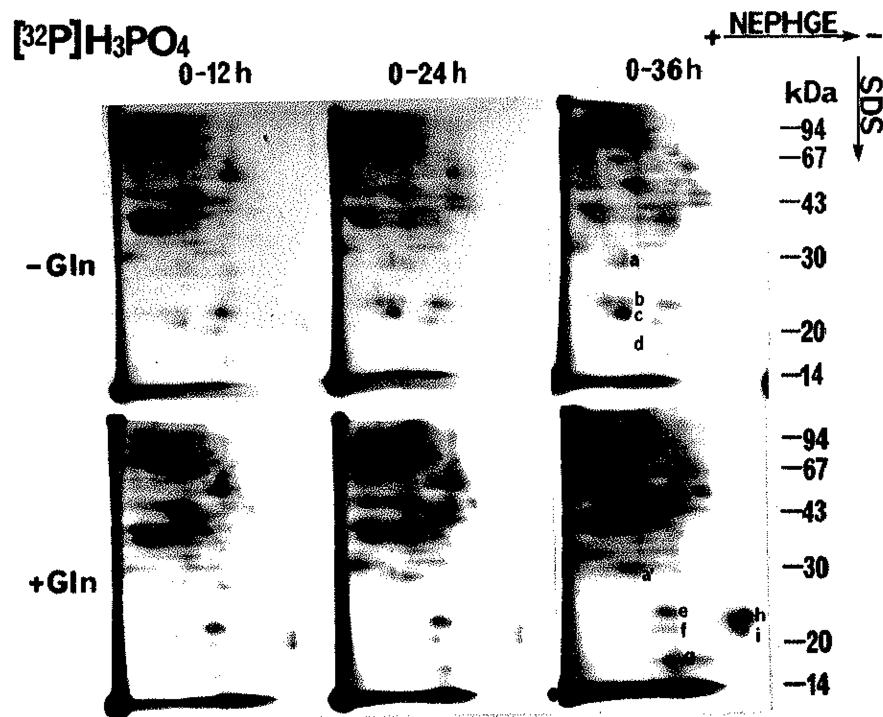

Fig. III-2. Changes in the electrophoretic patterns of phosphorylated proteins of pollen grains during dedifferentiation and maturation. Fraction III pollen grains were cultured in medium B' with additions of $^{32}P_i$ (10 μCi/ml) and glutamine at a concentration of 0 or 1 mM. Pollen grains were harvested after various periods of culture. Phosphorylated proteins from 2 x 10^5 (-glutamine) or 10^5 (+glutamine) pollen grains were analyzed by 2-dimensional gel electrophoresis and autoradiography which were performed by applying the method described in the **Materials and Methods** section.

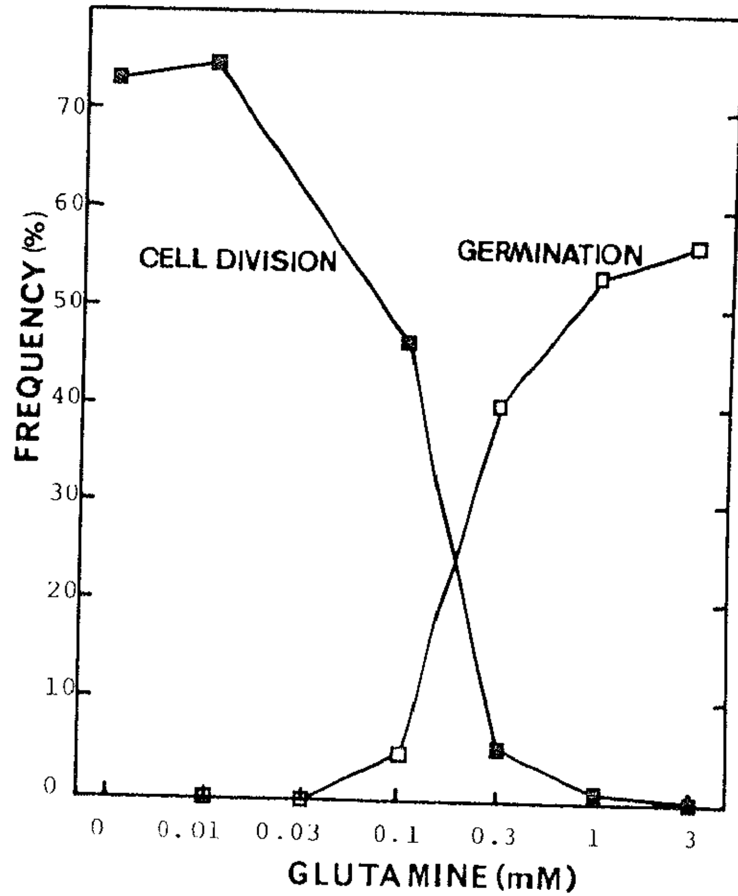

Fig. III-3. Effect of glutamine on the dedifferentiation (cell division) and maturation (germination) of pollen. Fraction III pollen was cultured in medium B' with addition of glutamine at various concentrations for 48 h and then transferred to medium C or D. Pollen cell division or germination was observed in the same manner as described in Chapter II.

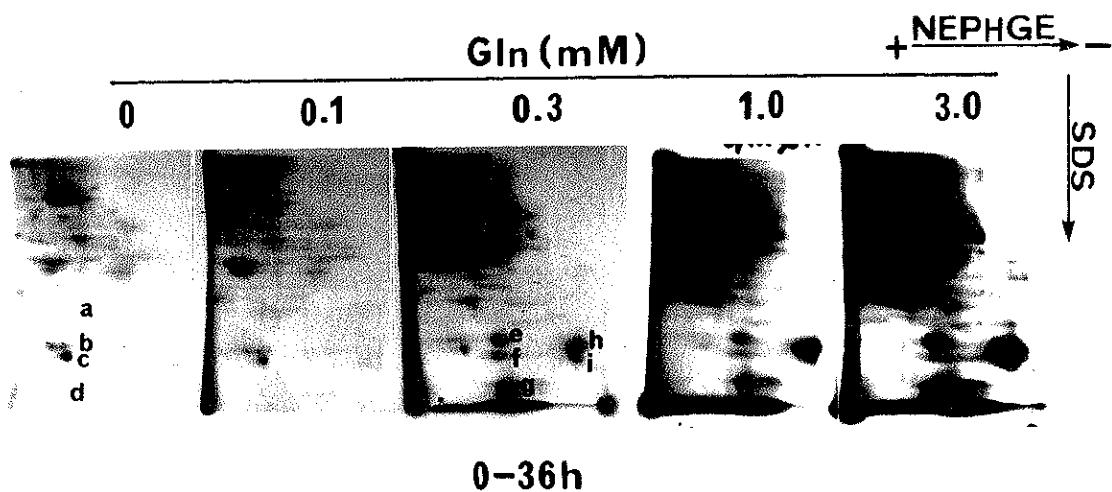

Fig. III-4. Effect of glutamine on the electrophoretic pattern of phosphorylated proteins. Fraction III pollen was cultured in medium B' with addition of $^{32}P_i$ (10 μCi/ml) and glutamine at various concentrations for 36 h. Phosphorylated proteins were separated by 2-dimensional gel electrophoresis (NEPHGE/SDS) and visualized by autoradiography. Protein prepared from 10^5 pollen grains was applied for each run.

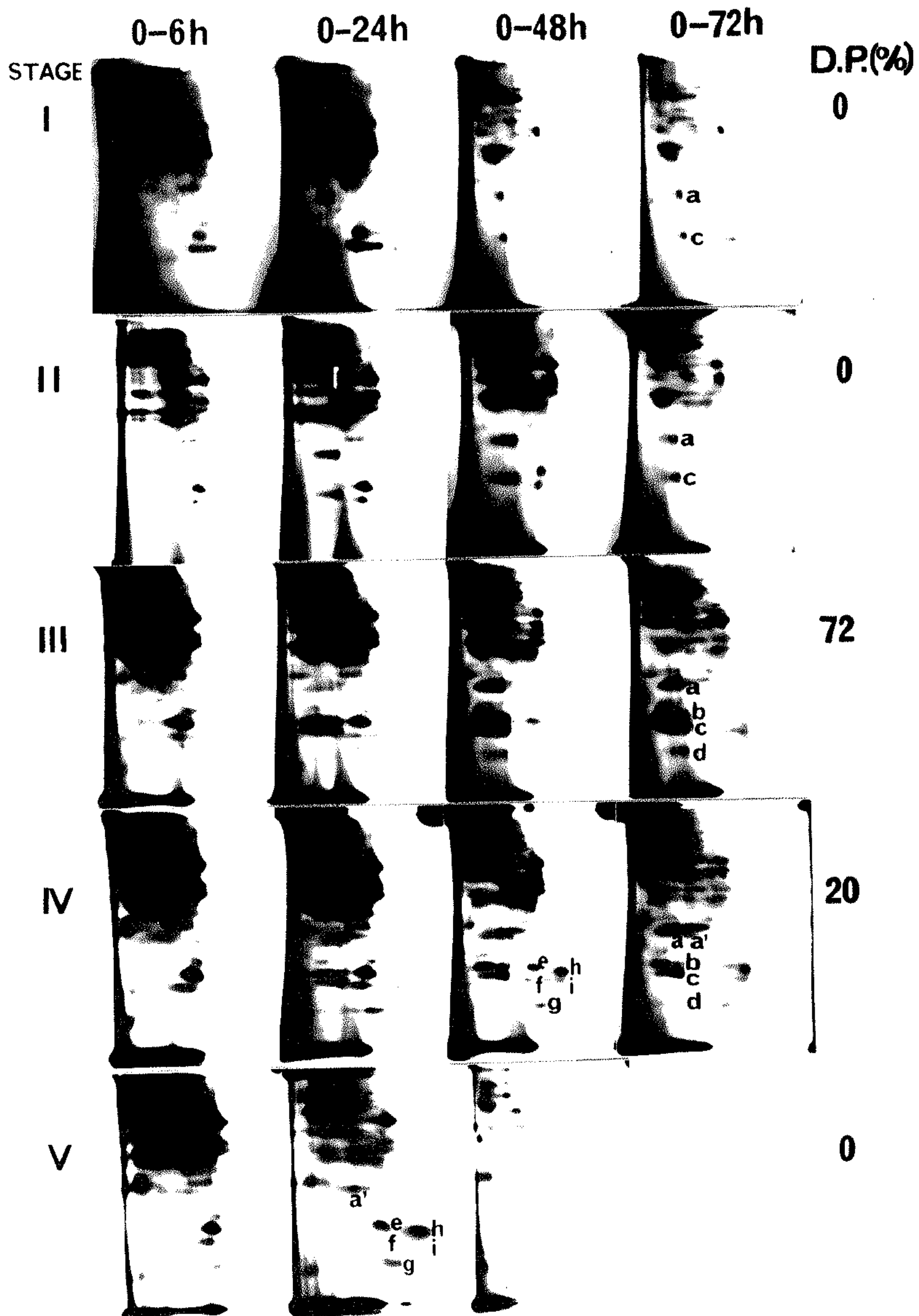

Fig. III-5. Changes in the electrophoretic pattern of protein phosphorylation of the pollen at various stages of culture in medium without glutamine. Four aliquots

of the suspension of pollen from each fraction were labelled in medium B" with $^{32}P_i$ (10 μCi/ml) for various periods and used for 2-dimensional gel electrophoresis as described in **Materials and Methods.** An aliquot of each suspension was put aside before labelling and cultured in medium B" for 72 h or 48 h (Fraction V) and then transferred to medium C. Cell division was observed by the method described in **Chapter II.**

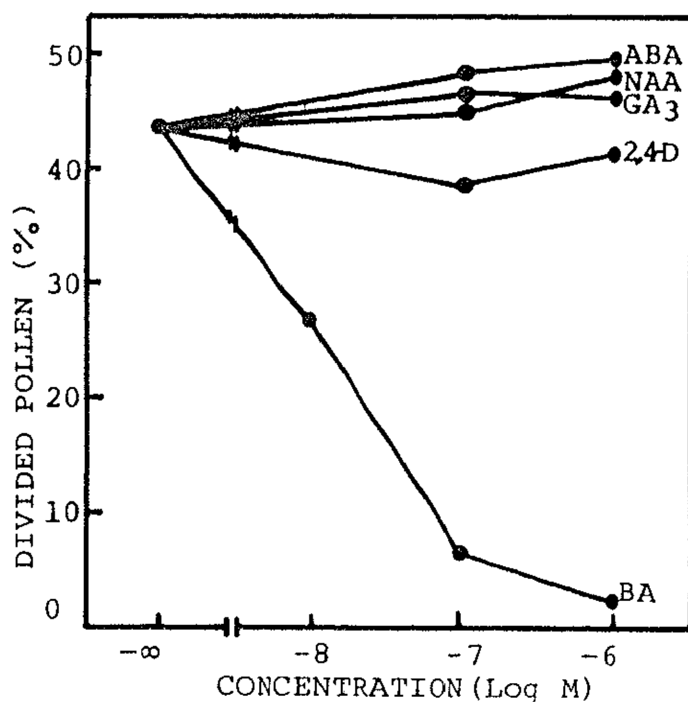

Fig. III-6. Effect of phytohormones on the dedifferentiation of pollen grains (divided pollen). Fraction III pollen grains were cultured in medium B" with the phytohormones indicated at various concentrations for 48 h and then transferred to medium C. Cell divisions were observed by the method described in **Chapter II.**

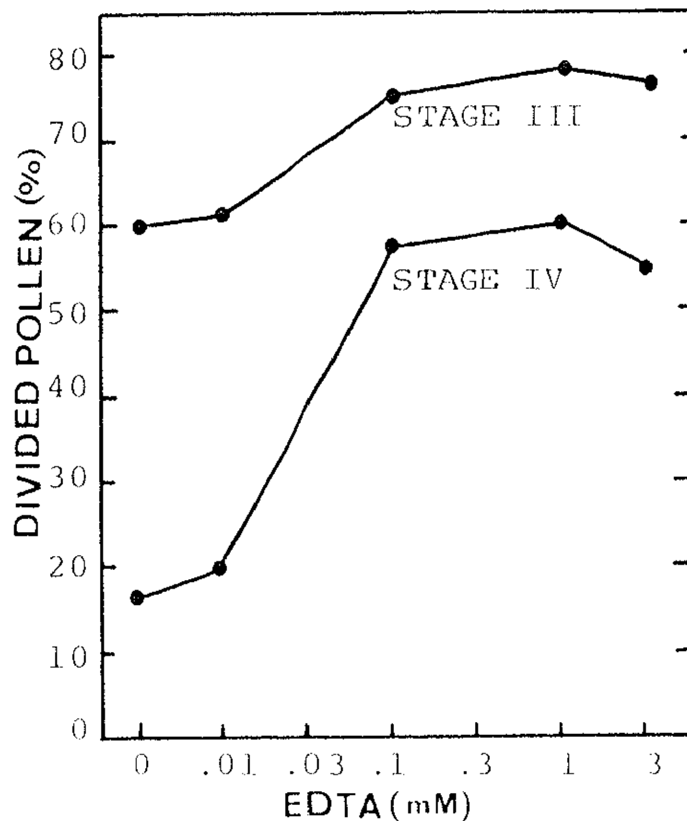

Fig. III-7. Effect of EDTA on the dedifferentiation of Fraction III and IV pollen (divided pollen). Fraction III or IV pollen were cultured in medium B" with EDTA at various concentrations for 48 h and then transferred to medium C. Cell divisions were observed by the method described in **Chapter II.**

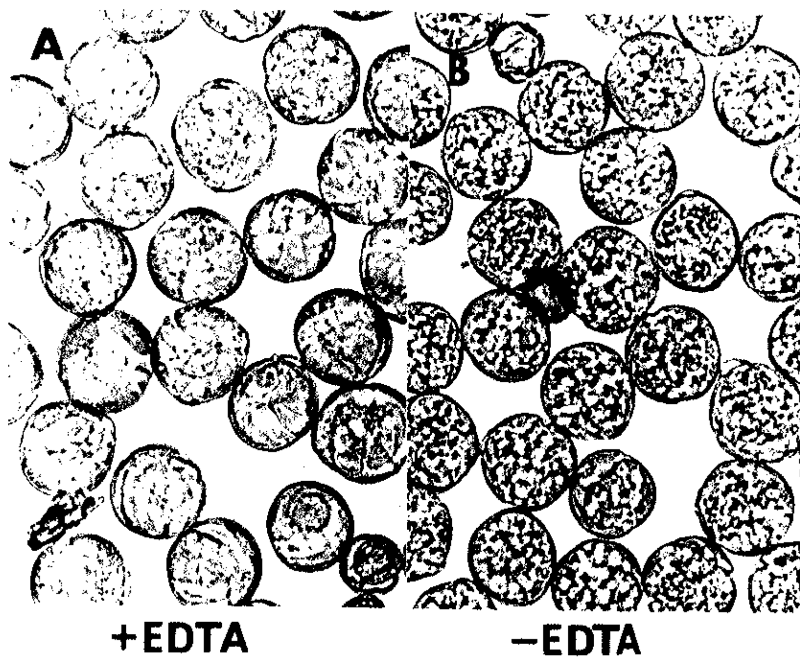

Fig. III-8. Aspects of Fraction IV pollen cultured for 48 h in medium B" with no (B) or 1 mM (A) EDTA.

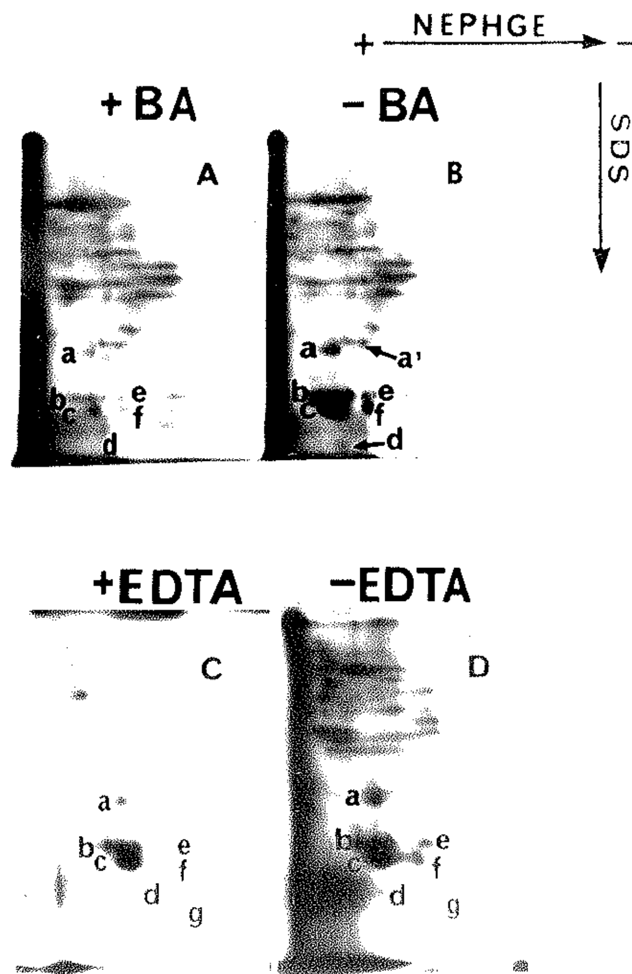

Fig. III-9. Effects of BA and EDTA on the electrophoretic pattern of phosphorylated proteins of Fraction III pollen. Fraction III pollen was labelled in medium B" with $^{32}P_i$ (10 μCi/ml) and BA 1 μM (A) or EDTA 1 mM (C) for 48 h. After labelling, proteins from each sample (10^5 grains) were analyzed by 2-dimensional gel electrophoresis as described in **Materials and Methods.** (C) and (D) were the control of (A) and (C), respectively.

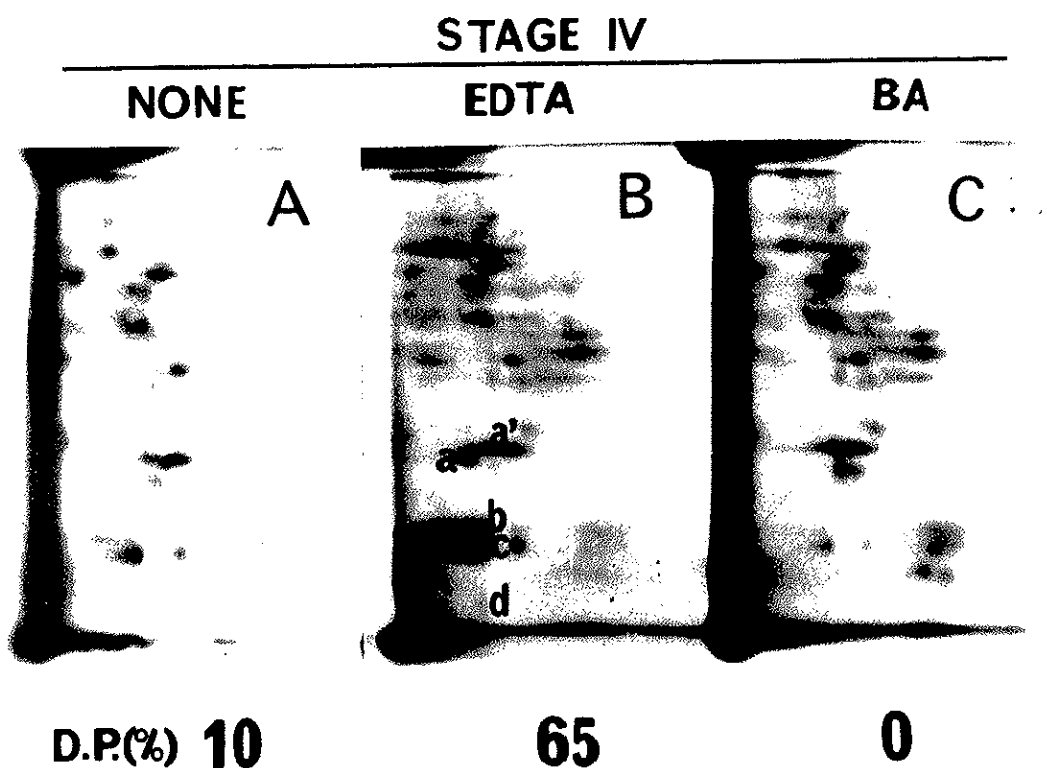

Fig. III-10. Effects of EDTA and BA on the pattern of phosphorylated proteins from Fraction IV pollen. Fraction IV pollen was suspended in medium B''(A) or in medium B'' with EDTA 1 mM (B) or BA 1 μM (C) in duplicate. $^{32}P_i$ was added to one each of the duplicates (10 μCi/ml). After 48 h the labelled samples were harvested and analyzed by 2-dimensional gel electrophoresis as described in **Materials and Methods.** The other duplicates were transferred to medium C, and cell divisions were observed as described in **Chapter II.** The figures indicated were the frequency of dedifferentiated pollen (divided pollen) observed in the 2nd culture.

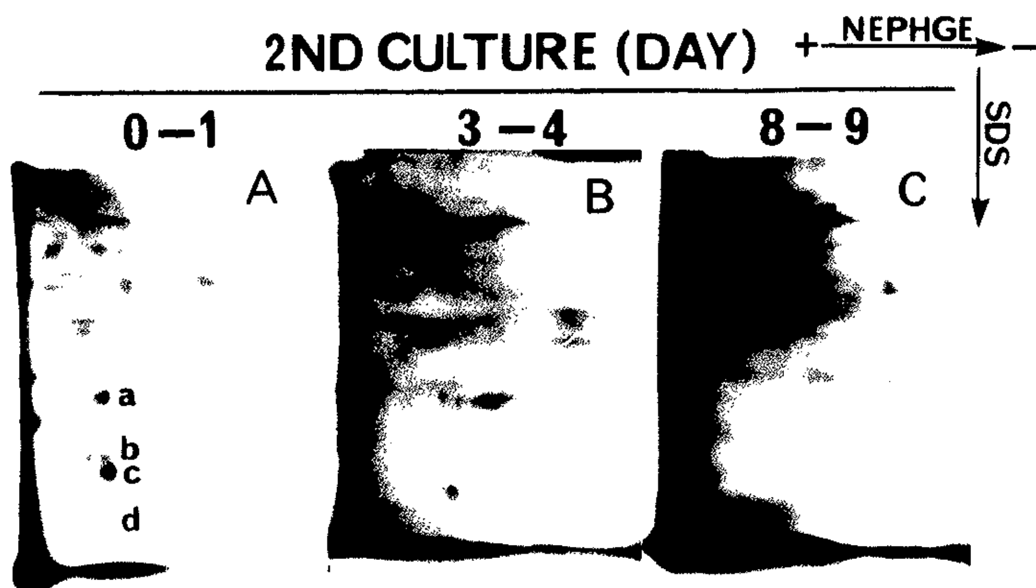

Fig. III-11. Changes in the electrophoretic pattern of phosphorylated proteins during the 2nd culture. Fraction III pollen grains were cultured in medium B" for 48 h and then transferred to medium C. After various periods of the 2nd culture, pollen grains were transferred to medium C" with $^{32}P_i$ (10 μCi/ml) and labelled for 24 h, i.e., during the 2nd culture day 0-1 (A), day 3-4 (B), day 8-9 (C). The samples were analyzed by 2-dimensional gel electrophoresis as described in **Materials and Methods.**

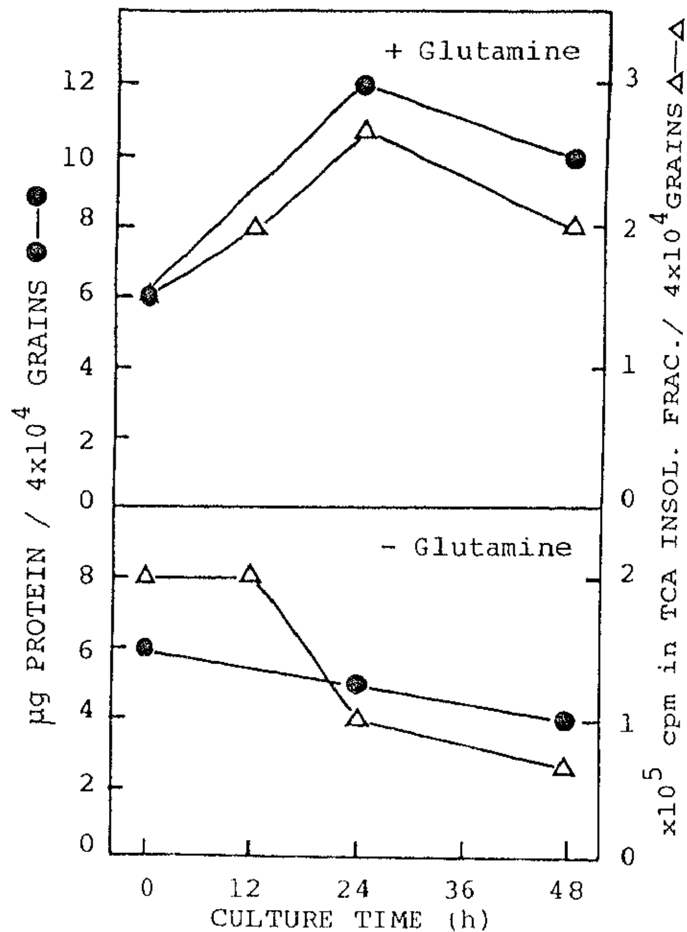

Fig. III-12. Changes in the protein contents and the rate of protein synthesis during the maturation and dedifferentiation of pollen. Fraction III pollen grains were cultured in medium A (+glutamine) or B (-glutamine). After 0, 21 and 45 h of culture, the labelling of the pollen grains was started by adding [^{35}S]-methionine into the medium (10 μCi/ml). Six h after the beginning of the labelling, the pollen grains (10^5/ml) were harvested and homogenized in 200 μl solution containing

0.2 M Tris-HCl (pH 7.0), 2% SDS, 5% 2-mercaptoethanol. Protein content of each sample was examined by Bio-rad protein assay system. The incorporation of radioactivity into acid insoluble fractions of each sample was examined as described below. An aliquot of each sample was mixed with 2 ml of 10% TCA. The precipitate was collected on Whatman GF/C glass filter paper, and thoroughly rinsed first with 5% TCA, and then with 70% ethanol. After drying, the radioactivity absorbed onto filter paper was counted with a Beckman liquid scintillation spectrometer (Model LS-250). The scintillation liquid consisted of 0.4% of 2,5-diphenyloxazole and 0.01% of 1,4-bis-2-(5-phenyloxazolyl)benzene in toluene.

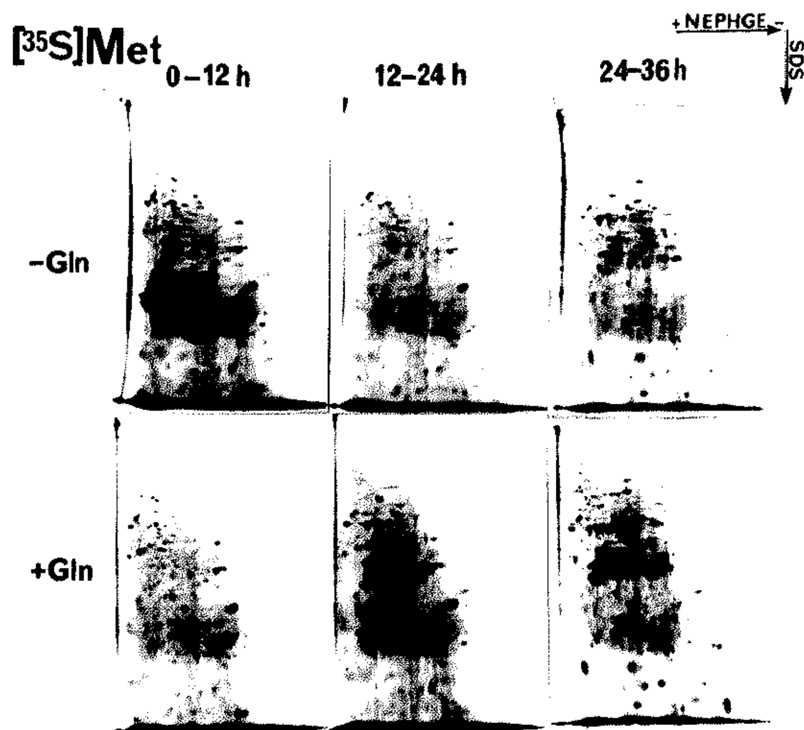

Fig. III-13. Changes in the electrophoretic pattern of newly synthesized proteins during the dedifferentiation and maturation of pollen. Fraction III pollen was cultured in medium A (+glutamine) or medium B (-glutamine). After 0,12 and 24 h, the labelling of pollen was started by adding [^{35}S]-methionine into the medium (10 μCi/ml). After 12 h, pollen grains were harvested. The labelled proteins in each sample were separated by 2-dimensional gel electrophoresis and visualized by autoradiography as described in the **Materials and Methods** section.

Concluding Remarks

In **Chapter I**, we presented a new method for inducing embryogenesis in a high percentage of Nicotiana rustica pollen. A highly homogeneous population of pollen grains at mid-binucleate stage was obtained by Percoll density gradient (35/45%) centrifugation of a pollen suspension prepared from buds with 6-9 mm corolla. We cultured these pollen grains in 0.4 M mannitol for 3 days (the first culture), then collected and transferred them to 0.4 M mannitol with MS salts, 40 mM galactose, 3 mM glutamine and 5 μM ABA, culturing it for 10 days (the 2nd culture). Maximally 70% of these pollen grains underwent cell division and 40% of the divided pollen grains formed well developed embryos during subsequent culturing in MS medium with 0.4 M mannitol and 3 mM glutamine. Without including the 1st culture period or with pollen at a developmental stage other than mid-binucleate, embryogenic cell division could not be induced. It was clear that both a specific developmental stage and a certain period of starvation are important for induction of pollen embryogenesis. However, it was difficult to attain in vitro maturation. Therefore, we could not clarify the main factor(s) that determine(s) the developmental pathway of immature pollen from gametophytic to sporophytic.

In **Chapter II**, we confirmed that both stage specificity and starvation period are also required for embryogenesis of pollen in Nicotiana tabacum. In that

species, we directed the in vitro developmental pathway of the pollen by regulating the glutamine concentration in the culture medium. When the highly homogeneous population of pollen grains at mid-binucleate stage (Stage III) was cultured in basal medium with addition of glutamine, most underwent normal maturation. On the other hand, if they were first cultured in only basal medium, most grains did not mature, but after transfer to medium with glutamine and sucrose, they began to divide. Evidently, essential changes necessary for the embryogenic response can be induced by glutamine starvation only in pollen grains at a specific developmental stage. We believe that this culture method, using a highly homogeneous cell population, will make it possible to study the mechanism of induction of pollen embryogenesis by the biochemical approach.

In **Chapter III**, we reported the reliable biochemical markers which appear when the immature pollen grains dedifferentiate to embryogenic cells. We examined the pattern of phosphorylated proteins of pollen by 2-dimensional gel electrophoresis and autoradiography, and found several phosphorylated proteins that appeared in pollen grains transforming to embryogenic cells in the medium without glutamine. The appearance of the phosphorylated proteins was in close relation with the culture time (after 24 h) and the presence of the inhibitors (glutamine and BA) or the promoter (EDTA) of pollen

dedifferentiation. We conclude that the protein phosphorylation is one of necessary steps for the dedifferentiation of pollen grains.

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