Embryogenic Induction in Pollen Grains of *Nicotiana tabacum* L.

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The process of embryogenic induction in pollen grains of tobacco was investigated through direct pollen culture. Pollen grains at the late unicellular to middle bicellular stages could develop into seedlings through a process of embryogenesis when they were cultured in sucrose-free medium for several days and then transferred into Nitsch's liquid medium containing sucrose, as described by GHANDIMATHI (1982) and IMAMURA *et al.* (1982). Based on microscopic observations of cultured pollen grains and experiments where ^3H -thymidine was incorporated into the pollen grains, it was revealed that pollen grains accumulated starch granules resulting in pollen germination and that DNA was synthesized only in the generative nucleus, suggesting that they continued their gametophytic development in the medium containing sucrose. On the other hand the gametophytic development was markedly suppressed in the sucrose free-medium. These findings indicate that the initial event of embryogenic induction is the suppression of the gametophytic development of the pollen grains and that embryogenic induction proceeds successfully in the absence of sucrose in the medium. Furthermore, the results of ^3H -thymidine incorporation showed that DNA synthesis in the vegetative nucleus, which was assumed to be the DNA synthesis leading to pollen embryogenesis, occurred soon after the suppression of the gametophytic development caused by the absence of sucrose. Since cultured cells generally do not undergo the phase of DNA synthesis when starved of sugar, it is inferred that pollen grains of tobacco have the inherent ability to synthesize DNA in the absence of sugar. It is concluded that the process of embryogenic induction in pollen embryogenesis consists of two phases, namely, the suppression of the gametophytic development caused by sugar starvation and DNA synthesis leading to pollen embryogenesis which proceeds depending on the inherent properties of pollen grains.

KEY WORDS : *Nicotiana tabacum*, pollen culture, pollen embryogenesis, embryogenic induction, DNA synthesis.

Introduction

Haploid plants have several advantage for plant breeding including the rapid production of homozygous lines as well as the possibility to detect directly recessive mutations. Since the first report by GUHA and MAHESHWARI (1966) on pollen embryogenesis with anther culture of *Datura innoxia*, anther culture and pollen culture have been considered to be useful methods for haploid production.

However, the number of plant species in which pollen embryoids have been formed by anther or pollen culture are not so many, most of these belonging to one family, namely the Solanaceae. In order to obtain pollen embryoids in a larger number of species, more intensive and analytical investigations on each process of pollen embryogenesis, especially on the process of embryogenic induction, are needed. However few physiological and biochemical investigations on the process of embryogenic induction have been carried out (VASIL 1980) presumably because analytical investigations are very difficult to perform when pollen embryogenesis is induced under the influence of anther culture. Recently, successful direct pollen culture without prior treatment of buds or prior culture

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of anthers was reported in *Nicotiana* species (GHANDIMATHI 1982, IMAMURA et al. 1982). According to these reports, pollen embryogenesis can be induced if sugar is absent in the culture medium. Pollen embryoids were obtained when pollen grains were cultured for several days in distilled water or in a medium containing only mineral salts prior to culture in nutrient medium. This method is very useful for the investigation of the induction of pollen embryogenesis.

In this paper, physiological changes of pollen grains from gametophytic development to pollen embryogenesis were analysed in conjunction with the effect of sugar starvation.

Materials and Methods

Nicotiana tabacum L. cv. Hicks 2 (supplied by the Japan Tobacco and Salt Public Corporation) was used for the experiments. Floral buds containing pollen grains at different developmental stages were collected from plants grown in a greenhouse. Immediately after the collection, they were surface sterilized and anthers were excised aseptically. Pollen grains were squeezed out from the anthers by pressing them with a glass stick in distilled water or in liquid medium. Fragments of anther tissues were removed by filtering the suspension through a nylon sieve of 80 μ m pore size. The pollen grains were then washed twice in distilled water or liquid medium by centrifugation at 500 rpm and suspended in distilled water or in Nitsch's medium containing mineral salts, 2% sucrose, 5000 mg/l of myo-inositol and amino acids (800 mg/l of glutamine, 100 mg/l of serine) (NITSCH 1974) for pollen culture at a density of $1-5 \times 10^4$ pollen grains/ml. Each experiment was carried out using 8 Petri-dishes (5.5 cm in diameter) which contained 2.5 ml of the suspension and was repeated twice. The cultures were placed under continuous illumination (2,000 lux) at a temperature of 27°C.

Pollen grains were mounted in a drop of fluorescein diacetate solution (HESLOP-HARRISON and HESLOP-HARRISON 1970) and observed under a fluorescence microscope (440-480 nm in wave length) to detect their viability.

Deoxyribonucleic acid (DNA) synthesis of pollen grains was detected by microautoradiography with ^3H -thymidine incorporation. To monitor DNA synthesis during the culture in Nitsch's medium, pollen grains were incubated in medium supplemented with 1.5 μ Ci/ml of $[6-^3\text{H}]$ -thymidine (s. a. 20 Ci/mmol) at 27°C. After 3, 6, and 11 days of incubation, they were fixed with ethanol: acetic acid (3 : 1v/v). The fixed pollen grains were stained with Feulgen reagent and they were dehydrated in alcohol series and then mounted on slide glasses. They were dipped into Sakura NR-M 2 liquid emulsion, exposed in the dark at 5°C for 3 weeks and developed in Konidol X for 5 min at 20°C.

In order to examine DNA synthesis in pollen grains cultured in distilled water, pollen grains which were cultured for various days were transferred into distilled water supplemented with 1.5 μ Ci/ml of $[6-^3\text{H}]$ -thymidine (s. a. 20 Ci/mmol) and incubated for 24 h at 27°C. They were then fixed and microautoradiographs were made through the same procedures as those mentioned above.

Results

1. Induction of pollen embryogenesis

As shown in Table 1, pollen grains at the stage corresponding to the onset of pollen mitosis to the middle bicellular stage (immediately before starch accumulation) developed into seedlings through a process of embryogenesis when they were cultured in distilled water for several days and then transferred to Nitsch's medium. But the number of seedlings per Petri-dish varied with the stages of pollen grains and periods of culture in distilled water. When the period of culture in distilled water was shorter (four to six days in the mitotic stage, two and three days in the early bicellular stage and one day in the middle bicellular stage), the process of embryogenesis somehow ceased at about

Table 1. Embryogenic induction from pollen grains at various stages of development by culture in distilled water

Developmental stage of pollen grains	Days of culture in distilled water											
	0	1	2	3	4	5	6	7	8	9	10	11
late unicellular	—	—	—	—	—	—	—	—	—	—	—	—
pollen mitosis	—	—	—	—	E	E	E	S (0.2)	S (0.2)	S (0.1)	—	—
early bicellular	—	—	E	E	S (0.5)	S (4.5)	S (50<)	S (50<)	S (9.6)	S (1.5)	S (0.2)	—
middle bicellular	—	E	S (0.2)	S (1.0)	S (6.5)	S (15.7)	S (50<)	S (50<)	S (8.9)	S (1.0)	S (0.2)	—
late bicellular	—	—	—	—	—	—	—	—	—	—	—	—

Note: Isolated pollen grains were cultured in distilled water for different period of time, then transferred into Nitsch's medium. S, E, —, indicate seedlings, embryoids in which the development was arrested at about the 20-celled stage, and no division of pollen grains observed 30 days after the beginning of the culture, respectively. Figures in brackets indicate the average number of seedlings per Petri-dish.

Table 2. Effects of components of Nitsch's medium on embryogenic induction in pollen grains

W	Composition of medium				Petri-dish with seedlings (%)	Number of seedlings per Petri-dish
	S	M	A	I		
○	○	○	○	○	0	0
○	○		○	○	0	0
○	○	○			0	0
○	○				0	0
○		○	○	○	37.5	3.1
○		○			95.0	50<
○			○		75.0	22.5
○				○	50.0	7.1
○					95.0	50<

Note: Components of medium; W: distilled water, S: sucrose, M: Mineral salts, A: amino acids (glutamine, serine), I: myo-inositol, Open circle indicates the addition of the component.

Pollen grains isolated at the early bicellular stage were cultured in various media for 7 days, then transferred into Nitsch's medium. Data were recorded 30 days after the beginning of the culture.

Table 3. Effects of sucrose on embryogenic induction in pollen grains

	Concentration of sucrose (%)						
	0	0.01	0.05	0.1	0.5	1	2
Embryogenic induction	+	+	—	—	—	—	—
Number of seedlings per Petri-dish	50<	17.5	—	—	—	—	—

Note: Pollen grains isolated at the early bicellular stage were cultured in media containing various sucrose concentrations for 7 days, then transferred into Nitsch's medium. Data were recorded 25 days after the beginning of the culture. +: embryogenesis was induced, —: not induced.

Fig. 1. Changes of rate of surviving pollen grains during the culture in distilled water.

the 20-celled stage in Nitsch's medium. The optimum period of culture in distilled water to obtain seedlings was approximately 7 days and many seedlings were formed from pollen grains which were both in the early and middle bicellular stages.

Pollen grains isolated at the late unicellular stage could not survive in distilled water since the osmotic pressure was inadequate (GHANDIMATHI 1982). If they were cultured in 0.1 M mannitol solution for seven days and then transferred to Nitsch's medium, embryo-ids and seedlings were formed (about 25 seedlings per Petri-dish).

In order to analyse the effects of each component of Nitsch's medium on embryogenic induction, pollen grains at the early bicellular stage were cultured in various media for seven days and then transferred to Nitsch's medium (Table 2). It was evident that pollen embryo-ids were formed only when pollen grains were precultured in sucrose-free media.

Fig. 2. Incorporation of ^3H -thymidine into pollen grains cultured in Nitsch's medium.

Note: Silver grains were observed in the generative nucleus of bicellular pollen grains (▽), germinated pollen grains and enlarged pollen grains (○), and dead pollen grains (▲). The percentage of the four kinds of pollen grains are totaled (●).

Fig. 3. Incorporation of ³H-thymidine into pollen grains cultured in distilled water.

Note: Open bar, striped bar and dotted bar indicate the percentage of pollen grains in which silver grains were observed in the generative nucleus, vegetative nucleus and both nuclei, respectively.

Fig. 4. Cell division of pollen grains after transfer into Nitsch's medium from distilled water.

Note: Pollen grains were transferred into Nitsch's medium after 2 (▼), 6 (●) and 9 (○) days of culture in distilled water.

Table 3 shows the effects of sucrose on embryogenic induction. It was observed that very small amounts of sucrose (0.05%) inhibited the induction of embryogenesis.

Based on the microscopic observations of cultured pollen grains it was revealed that, when pollen grains isolated at the early bicellular stage were cultured in Nitsch's medium, a marked decline in the number of surviving pollen grains occurred and surviving pollen grains accumulated starch granules. Some of them germinated and the others markedly enlarged after about three to five days of culture. Starch accumulation in pollen grains was always observed when they were cultured in various media containing sucrose. These observations suggest that pollen grains continue their gametophytic development when cultured in media with sucrose.

On the other hand, when pollen grains at the early bicellular stage were cultured in distilled water, the number of surviving pollen grains decreased gradually until the 14th day of culture at which no surviving pollen grain was observed (Fig. 1). Neither starch accumulation in the pollen grains, pollen germination, nor development into enlarged pollen grains was observed when the pollen grains were cultured in distilled water or transferred to Nitsch's medium after more than five days of culture in distilled water. Furthermore whenever pollen grains were cultured in various media lacking sucrose, starch accumulation in pollen grains was never observed. These observations suggest that the gametophytic development of pollen grains is inhibited in sucrose-free media.

2. DNA synthesis in cultured pollen grains

Pollen grains isolated at the stage of early bicellular were cultured in Nitsch's medium with ^3H -thymidine (Fig. 2). Only the generative nucleus incorporated ^3H -thymidine and the greater part of incorporation occurred within 3 days after the inoculation. Subsequently, most of the pollen grains which incorporated ^3H -thymidine either germinated or became enlarge pollen grains. In intact pollen grains of tobacco plants, it has been observed that DNA is synthesized in the generative nucleus immediately after pollen mitosis and that DNA synthesis in the vegetative nucleus never occurs (ARUGA *et al.* 1982). Therefore, it is reasonable to assume that the observed incorporation of ^3H -thymidine corresponded to DNA synthesis which occurred during the gametophytic development. Therefore, it is concluded that pollen grains continue their gametophytic development when they are cultured in Nitsch's medium.

Fig. 3 shows the results of the experiment on the incorporation of ^3H -thymidine using pollen grains isolated at the early bicellular stage and cultured in distilled water. It was evident that DNA was actively synthesized in distilled water. Furthermore, incorporation of ^3H -thymidine into the vegetative nucleus which never occurs during the gametophytic development of pollen grains was observed.

But as far as the pollen grains were cultured in distilled water, they did not undergo cell division. Their cell cycle was arrested in the G_2 phase. When pollen grains which had synthesized DNA were transferred to Nitsch's medium, cell division start at once (Fig. 4) and pollen grains developed into embryoids.

Discussion

From the present experiments, it was evident that pollen embryogenesis in tobacco could be successfully induced by culture of pollen grains directly in sucrose-free media for several days. The results confirmed the view that absence of sugar in the medium leads to the induction of pollen embryogenesis in *Nicotiana tabacum* (GHANDIMATHI 1982) and *N. rustica* (IMAMURA *et al.* 1982).

From the microscopic observations and results of ^3H -thymidine incorporation, it was revealed that pollen grains continued their gametophytic development in Nitsch's medium containing sucrose, while in the medium lacking sucrose gametophytic development of pollen grains was prevented. BHOJWANI *et al.* (1973) reported that the RNA and protein contents of pollen grains of tobacco were reduced in the inductive period of embryogenesis whereas they increased in gametophytic development of pollen grains. Based on electron microscopic observations, DUNWELL and SUNDERLAND (1974) showed that cell organella of embryogenic pollen grains underwent degenerative changes during the inductive period. Similarly, in embryogenic pollen grains of *Datura innoxia*, prevention of gametophytic development, which was indicated by the suppression of starch and RNA accumulation and organella development, was observed at the beginning of anther culture (SANGWAN-NORREEL 1978). On the basis of these findings, it can be considered that the initial event of embryogenic induction is suppression of gametophytic development of pollen grains.

NITSCH and NORREEL (1973) showed that cold treatment of buds before anther culutre

enhanced pollen embryogenesis. Furthermore, it was reported that short term treatment with reduced atmospheric pressure, anaerobic treatment of anthers immediately before inoculation and addition of abscisic acid to the culture medium during the initial one day of anther culture stimulated pollen embryogenesis (IMAMURA and HARADA 1980 a, b, 1981, HARADA and IMAMURA 1982). Since these treatments are known to inhibit development, it is inferred that these treatments suppress the gametophytic development of pollen grains either directly or through the prevention of the development of anther tissues, resulting in the enhancement of embryogenesis in anther culture. As mentioned above, sugar starvation blocked the gametophytic development of pollen grains drastically. Therefore, it is considered that the role of sugar starvation on embryogenic induction is suppression of the gametophytic development of pollen grains. Chemical analysis on anther tissues of tobacco showed that only very small amounts of soluble sugars were present in the cultured anthers during the initial several days of anther culture (ARUGA and NAKAJIMA, data unpublished). This observation suggests that the induction of pollen embryogenesis in anther culture occurred by the same mechanism as that described above.

Besides, the present results indicated that ^3H -thymidine incorporation into vegetative nuclei occurred during the culture in distilled water following the suppression of the gametophytic development. As reported previously, DNA synthesis in the vegetative nuclei never occurred during gametophytic development of pollen grains (ARUGA *et al.* 1982), and pollen embryoids were formed through repeated divisions of the vegetative cells (SUNDERLAND and DUNWELL 1974), the incorporation of ^3H -thymidine into the vegetative nuclei observed here was inferred to be new DNA synthesis leading to pollen embryogenesis. Thus it is evident that pollen grains of tobacco were able to synthesize DNA leading to pollen embryogenesis in the absence of sugar. VAN'T HOF (1974) reported that cultured cells did not undergo the phase of DNA synthesis when starved of sugar. In this regard, the results of the present experiments is an unique case. Hence, it is suggested that pollen grains of tobacco have an inherent ability to synthesize new DNA when the gametophytic development is suppressed as a result of sugar starvation.

Finally, it is concluded that the process of embryogenic induction of pollen grains consists of two phases, namely, the suppression of gametophytic development and DNA synthesis leading to pollen embryogenesis. It is also concluded that the suppression of gametophytic development is caused by sugar starvation and that DNA synthesis proceeds depending on physiological properties inherent to the pollen grains. Further studies should be carried out to define the properties that enable pollen grains to synthesize DNA after the suppression of gametophytic development, in order to establish techniques for the production of haploid plants through pollen embryogenesis in a larger number of species.

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タバコにおける花粉からの不定胚発生の誘導

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タバコにおける花粉からの不定胚発生の誘導過程を, GHANDIMATHI (1982) および IMAMURA *et al.* (1982) によって報告された花粉の直接培養法を用いて研究した.

1細胞期後期から2細胞期中期までの发育段階にある花粉は, 蔗糖を添加しない培地で数日間培養した後 NITSCH (1974) の培地に移植することによって, 不定胚発生を経て, 発芽個体へと发育した. 花粉を直接蔗糖を添加した培地で培養すると, 花粉における澱粉粒の蓄積, 発芽, 生殖核のみへの ^3H -thymidine のとりこみなどが観察され, 生存花粉では正常な发育が進行していることが明らかとなった. 一方, 花粉を蔗糖を添加しない培地で培養すると, 花粉の正常发育は完全に停止した. これらの結果から, 花粉からの不定胚発生の誘導過程における最初の段階は, 花粉の正常发育の停止であり, この段階は糖飢餓によって効果的に進行するものと考えられた. さらに, ^3H -thymidine のとりこみの結果から, 花粉は糖飢餓下において, 不定胚発生に向うものと考えられる栄養核における DNA 合成を行なっていることが明らかとなった. 培養細胞は通常, 糖飢餓

下においては新たな DNA 合成期にははいらないことが知られている (VAN'T HOF 1974). 従って, タバコの花粉は糖飢餓下において, 正常発育の停止にひきつづいて, 新たな DNA を合成し得る特殊な生理的条件をそなえていたものと考えられた.

以上の結果から, タバコにおける花粉からの不定胚発生の誘導過程は, 糖飢餓によってひきおこされる正常発育の停止と, 花粉の内生的な条件に依存して進行する不定胚発生への新たな DNA 合成との2つの段階から成ると結論された.