Isolation of Living Pollen Mother Cells in *Brassica* Species and Extraction of mRNA from PMCs*

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Summary

The first step of clonings of genes expressed in pollen mother cells (PMCs) is to isolate living PMCs. In the procedure of isolating PMCs from cabbage (*Brassica oleracea* var. *capitata*) and Chinese cabbage (*B. campestris* ssp. *pekinensis*), 1%~30% sucrose, 2%~10% mannitol, CPW medium (Frearson et al., 1973) and B5 medium (Gamborg et al., 1968) were used to maintain the cell vitality. The result showed that 7% sucrose was better in maintaining the cell vitality of PMCs. Percoll gradient density centrifugation was used to separate PMCs from tetra stage microspores and unicellular microspores by two steps. The first steps, 0% / 10% percoll, can separate unicellular microspores from PMCs and tetra microspores; the second step, 0~10% / 20% percoll, could isolate PMCs from tetra microspores. The isolation of PMCs of cabbage was effective than Chinese cabbage. The 0.7mg PMCs and 3.3mg tetra microspores were obtained from one gram buds. The total RNA was extracted from PMCs and tetra microspores. There were two major ribosomal RNA bands (18S rRNA and 28S rRNA) after total RNA agarose gel electrophoresis. The poly(A)+ RNA was purified from total RNA by the way of oligo(dT)-cellulose affinity column chromatography.

Key Words: *Brassica* spp., pollen mother cell, isolation, mRNA

Introduction

The development of male gametophyte in angiosperms plays a key role in sex reproduction (Masca-renhas, 1990). Recently, many genes expressed specifically or preferentially in male gametophyte have been cloned, however, these genes were mainly microspore-specific or pollen-specific (Kamalay and Goldberg, 1980; Kim et al., 1994; MacCormick, 1991; Roberts et al., 1991; Stinson et al., 1987; Twell et al., 1989; Weterings et al., 1995; Wing et al., 1989). Since meiosis marks the transition from sporophyte to gametophyte, there are many genes functioning during meiosis. The isolation of these genes expressed specifically in PMCs (PMC) or meiocytes will be of importance in understanding of meiosis process.

PMCs only occupy a very small part of anthers or buds. Some mRNAs of PMC-specific genes may be in low abundance, especially the rare mRNAs. Consequently, it may be difficult to isolate the cDNAs of PMC-specific genes by differential screening of cDNA libraries made from anthers or buds at meiotic phases. In

order to clone PMC-specific genes, we try to isolate PMCs and to make a cDNA library of mRNAs extracted directly from PMCs.

Materials and Methods

Plant materials include cabbage (*Brassica oleracea* var. *capitata*) and Chinese cabbage (*B. campestris* ssp. *pekinensis*). Flower buds were carefully selected after checking the developmental stages under a microscope. The flower buds at meiotic stages were chosen and crashed in a test tube with a glass pestle to release PMCs in different solutions of sucrose, mannitol, B5 medium or CPW medium. The homogenate was filtered through a 30-50m steel mesh, and centrifuged at 500rpm for 5 min to collect PMCs. The PMCs were separated from other cells through percoll gradient density centrifugation two times. The first time 10%(v/v) percoll solution was used. Then 20% percoll was used. All procedures above were finished in ice box or at low temperatures (5 °C) as quickly as possible. After separation, PMCs and tetra stage micropores were put into liquid nitrogen immediately.

The isolation of total RNA from PMCs, tetra microspores and unicellular microspores, respectively, was made according to Bowsher's way (Bowsher, 1991). Then mRNA was purified from total RNA using the oligo(dT)-cellulose affinity column chromatography.

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Results

Determination of solutions for isolating living PMCs

The objective of the isolation of PMCs was to extract mRNA from PMCs. It was important to obtain living PMCs for the isolation of undegraded mRNA. One ~30%(w/v) sucrose solution, 2~10% mannitol solution and CPW and B5 media were used to isolate PMCs (Table 1). The cell features varied considerably after homogenization of flower buds; three kinds of male gametophyte could be clearly observed: normal cells, vacant cells and plasmolysed cells. The normal cells had the cell contents distributed within the cell evenly. Most

of these cells were living. The vacant cells lacked their nuclei and were dead. The third group of cells became plasmolysed due to high osmotic pressure of the solution. In lower sucrose solutions, the percentage of living cells was low, and in 30% of sucrose solution, all living cells were plasmolysed. Seven~20% of sucrose appeared to maintain high percentage of living cells.

When mannitol was used, the percentage of normal cells was as high as that in sucrose, but the percentage of living cells was lower than that in sucrose.

CPW medium has commonly used in plant protoplast isolation and B5 medium has often used in isolated microspore culture. However, plasmolysis of PMCs occurred in both media in the present study.

Table 1. Characteristics of PMCs of *Brassica campestris* in different solutions.

Solutions	Cell types of PMCs after homegenization			Cell vitality (%) ^z
	Normal cell(%)	Vacant cell(%)	Plasmolyzed cell(%)	
1% sucrose	80~90	10~20	0	30
4% sucrose	80~90	10~20	0	60
7% sucrose	80~90	10~20	0	70
10% sucrose	80~90	10~20	0	70
15% sucrose	80~90	10~20	0	70
20% sucrose	80~90	10~20	0	70
30% sucrose	0	10~20	80~90	—
2% mannitol	80~90	10~20	0	50
5% mannitol	80~90	10~20	0	60
10% mannitol	80~90	10~20	0	60
CPW medium	0	10~20	80~90	—
B5 medium	0	10~20	80~90	—

^z Living cells determined by plasmolysis (%) in high osmotic solution(30% sucrose).

Table 2. Efficiency of isolation of PMCs in *Brassica* spp.

Material	Percoll gradient density centrifugation	Cell layer	Cell type	Cell components	Cell vitality (%) ^z
<i>B. campestris</i>	1st	upper	100 % microspores	unicellular ^y	70 %
		bottom	80 % normal cells+ 20 % vacant cells	tetrad+PMC	70 %
	2nd	upper	70~80 % PMC+ 20~30 % tetrad	PMC+tetrad	70 %
		bottom	70~80 % tetrad+ 20~30 % PMC	tetrad+PMC	70 %
<i>B. oleracea</i>	1st	upper	100 % microspores	unicellular	70~80 %
		bottom	80 % normal cells+ 20 % vacant cells	tetrad+PMC	70~80 %
	2nd	upper	80~90 % PMC+ 10~20 % tetrad	PMC+tetrad	70~80 %
		bottom	80~90 % tetrad+ 10~20 % PMC	tetrad+PMC	70~80 %

^z Tested by plasmolysis, ^y unicellular microspores.

Table 3. Percentage of male gametophytic cells in flower buds of *Brassica* based on quantity.

Flower buds used(g)	Developmental stage	Male gametophytic cells (mg)	Percentage of male gametophytic cells in buds
3	Meiotic stage	2	0.07
3	Tetrad stage	10	0.33
3	Early unicellular stage	94	3.0
3	Later unicellular and bicellular stage	128	4.3

The procedure of separating PMCs from microspores by percoll gradient density centrifugation included three steps. First step was to collect all male gametophytic cells at a speed of 500rpm for 5 min. The second step was to put the cell suspension on the top of 10% percoll solution. In this step, the separation was very efficient and nearly all unicellular microspores were separated from PMCs and tetrad stage microspores in both *B. campestris* and *B. oleracea* (Table 2.). Finally, PMCs were separated from tetrad stage microspores by putting the solution on the top of 20% percoll solution. The results demonstrated that the separation of PMCs from tetrad stage microspores was easier in *B. oleracea* than that in *B. campestris*. In *B. oleracea*, the purity of PMCs was 80~90% and the remaining part was tetrad microspores, while in *B. campestris*, 70~80% of PMCs was obtained.

Percentage of male gametophytes in flower buds of Brassica campestris

Using flower buds at various developmental stages in *Brassica campestris*, percentage of male gametophytes was estimated through isolation (Table 3). The results showed that PMCs and tetrad stage microspores occupied 0.4% of flower buds, and PMCs consisted of only 0.07% of flower buds based on quantity of isolated PMCs. The percentage of microspores at early unicellular stage was about 3% of total flower buds, and about 5% of cells were pollen in flower buds based on the quantity of isolated microspores or pollen. Therefore, it was easier to isolate microspores than PMCs.

Isolation of total RNA and mRNA

After isolating total RNA from PMCs, tetrad microspores and unicellular microspores, OD260/OD280 value was ranged from 1.70 to 1.80, respectively. There were two major ribosomal RNA bands (18S rRNA and 28S rRNA) after total RNA agarose gel electrophoresis. The poly(A)⁺ RNA could be obtained from total RNA fractions by the way of oligo(dT)-cellulose affinity column chromatography.

Discussion

In the previous reports, the number of genes being active in mature pollen has been estimated in several plant species (Willing et al., 1988; Willing and Mascarenhas, 1984). The mRNAs have been divided into three

abundance classes: the highly abundant, the medium abundant and the least abundant. Most genes in pollen which consisted of 18,000 genes in *Tradescantia* and 17,000 genes in maize belong to the least abundant class presenting at an average of 0.001%. If the similar pattern occurs in PMCs and cDNA libraries made from mRNAs of flower buds, it will be very difficult to obtain cDNA clones of PMC-expressed mRNAs which belong to the least abundant class. The results in this report indicated that PMCs consisted of only 0.07% of flower buds selected. If the abundance of a mRNA is less than 0.001% in the PMC mRNAs, it is present only at 10^{-8} frequency. If mRNAs are extracted from flower buds, screening of at least 108 cDNA clones may be needed in order to isolate one cDNA clone. However, in most laboratories, the differential screening was carried out at a level of 105–106 cDNA clones. So, it is important to increase the abundance of mRNAs belonging to the least abundant class for the isolation of some cDNA clones.

The objective of the isolation of PMCs is to increase the abundance of mRNAs expressed specifically or preferentially in PMCs. For mRNA isolation, living cells are of importance in isolating undegraded mRNAs. Our study demonstrated 7~15% of sucrose concentrations were proper for the isolation of viable PMCs. The isolation of PMCs was efficient if percoll gradient density centrifugation was used. In this study, the purity of PMCs reached to 90% in *B. oleracea* and 80% in *B. campestris*. This result may be feasible for extracting undegraded mRNAs. Our following work will focus on the isolation of some PMC-specific cDNA clones which will be useful for understanding of molecular biology in meiosis.

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