

Male gametic cell-specific gene expression in flowering plants

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ABSTRACT The role of the male gamete—the sperm cell—in the process of fertilization is to recognize, adhere to, and fuse with the female gamete. These highly specialized functions are expected to be controlled by activation of a unique set of genes. However, male gametic cells traditionally have been regarded as transcriptionally quiescent because of highly condensed chromatin and a very reduced amount of cytoplasm. Here, we provide evidence for male gamete-specific gene expression in flowering plants. We identified and characterized a gene, *LGCI*, which was shown to be expressed exclusively in the male gametic cells. The gene product of *LGCI* was localized at the surface of male gametic cells, suggesting a possible role in sperm–egg interactions. These findings represent an important step toward defining the molecular mechanisms of male gamete development and the cellular processes involved in fertilization of flowering plants.

The process of male gametic cell development in higher plants begins with a highly asymmetric mitotic division of the male gametophyte (pollen). This division results in the formation of two unequal cells—the larger vegetative and the smaller generative cell—which have dramatically different structures and functions. The small generative cell is wholly enclosed within the much larger vegetative cell, forming a unique “cell-within-a-cell” structure. The generative cell, the progenitor of the male gametes (or sperm cells), has a very reduced amount of cytoplasm containing relatively few organelles and is surrounded by a double membrane. This cell undergoes a mitotic division producing two sperm cells. In some plants, such as maize, this division takes place in the mature pollen, and, in other plants, such as tobacco and lily, it occurs after pollination inside the pollen tube. One of the two sperm cells resulting from this division fertilizes the egg cell, and the other fuses with the central cell to produce the endosperm. In contrast, the larger vegetative cell comprises the major part of the pollen, including the pollen cytoplasm and the bulk of stored mRNAs, proteins, lipids, and polysaccharides. It is enclosed by a cellulosic inner wall, the intine, and a highly sculptured thick outer sporopollenin wall, the exine. During pollen germination, the vegetative cell wall extends, producing a pollen tube through which the two sperm cells ultimately are delivered to the female gamete.

The structural and functional differences between the vegetative and generative cells are likely to be controlled by cell-specific gene activity. Indeed, a number of vegetative cell-specific genes have been isolated, and, in some cases, a biological function has been assigned (1). For example, recently, a pollen-specific gene, *Zea m1*, was found to encode a protein involved in loosening the cell walls of the stigma and style and facilitating invasion of the pollen tube into the maternal tissues (2). In contrast, little is known about transcriptional activity of the cell of the male germline: the

generative and sperm cells. These cells are known to have highly condensed chromatin leading to an assumption that they are transcriptionally quiescent (3). It was further proposed that gametic cells are metabolically passive and wholly depend on the vegetative cell for their development and function (4).

Biochemical and molecular analysis of the male gametic cells has been hampered by the inaccessibility of these cells. Recently, researchers were able to isolate a sufficient number of generative cells from lily (*Lilium longiflorum*) pollen for biochemical analysis by using an enzymatic procedure (5). Metabolic labeling with ³⁵S methionine demonstrated that generative cells possess their own set of mRNAs and are capable of synthesizing proteins independently from the vegetative cells (5). Comparative analysis of protein profiles of generative cells and pollen grains showed the presence of common as well as cell-specific proteins in the generative cell. Here, we report the identification and characterization of a male gamete-specific gene, *LGCI*, of flowering plants. This study will facilitate our understanding of the molecular aspects of the male gamete differentiation and function in flowering plants.

MATERIALS AND METHODS

Construction and Screening of a cDNA Library. Generative cells were isolated from lily pollen as described (5) and were stored at -70°C until use. mRNA extracted from $\approx 1 \times 10^5$ stored generative cells by using a mRNA purification kit (Amersham Pharmacia) was reverse transcribed. The resultant cDNA was amplified by PCR and was size fractionated and cloned into the $\lambda\text{gt}11$ expression vector.

Inserts of cDNA clones randomly picked from the generative cell cDNA library were labeled with ³²P by random priming (Bresatec, Adelaide, Australia) and were used for probing of RNA slot blots that contained ≈ 300 ng of mRNAs from various tissues, including leaf, stem, petal, stigma/style, ovary, pollen, and generative cells. cDNA clones showing preferential or specific hybridization to generative cell mRNA were subcloned into pBluescript SK(+) vector (Stratagene) for further analysis.

Sequence Analysis. DNA sequencing was performed on both strands by the dideoxy chain-termination method by using an Applied Biosystems PRISM dye terminator cycle sequencing kit with an automated DNA sequencer. DNA sequence analysis was performed with DNA STRIDER software and the BLAST network service at the National Centre for Biotechnology Information (National Institutes of Health). A hydrophathy profile of *LGCI* protein was obtained according to Kyte and Doolittle (6). Secondary structure prediction was done according to Chou and Fasman (7).

RNA Gel Blot and Reverse Transcription (RT)–PCR Analysis. Total RNA was isolated from generative cells and various

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Abbreviations: RT, reverse transcription; DIG, digoxigenin.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF110779).

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tissues by using the SNAP RNA purification kit (Invitrogen). Ten micrograms of total RNA were separated on a 1% agarose gel containing formaldehyde, were transferred to a Hybond N⁺ nylon membrane (Amersham Pharmacia), and were probed with a ³²P-labeled *LGCI* cDNA insert. Hybridization was performed under high stringency conditions as described by Xu *et al.* (8). Filters subsequently were probed with lily ribosomal RNA to verify the amount of total RNA loaded from each tissue.

For RT-PCR, mRNAs from generative cells and various tissues were reverse transcribed and amplified by PCR with a pair of sequence-specific primers by using the Access RT-PCR System (Promega). For each tissue, mRNA was subjected to serial 2-fold dilutions. Based on the signal intensity of the amplified products, the relative amount of *LGCI* mRNA in each tissue was estimated.

In Situ Hybridization. Nonradioactive whole mount *in situ* hybridization was performed in both developing and mature pollen based on published protocols (9–11). Fresh pollen at various developmental stages was fixed [1% glutaraldehyde in 50 mM Pipes buffer (pH 7.4)] for 2 h at room temperature. The fixed pollen was washed in buffer and was stored in 70% ethanol at 4°C until use. Both sense and antisense riboprobes labeled with digoxigenin (DIG)-UTP were generated from linearized DNA templates. Hybridization signal was detected with an alkaline phosphatase-conjugated anti-DIG antibody by using a DIG nucleic acid detection kit (Boehringer Mannheim). To obtain better resolution, protoplasts of developing pollen were released from the exine by treatment with an enzyme solution (1% macerozyme/0.5% cellulase/0.5% BSA) as described (5). Vegetative and generative nuclei within the pollen were visualized by counter-staining with 4', 6'-diamidino-2-phenylindole.

In lily, generative cell division occurs in the pollen tube during its growth in the female stylar tissue. *In situ* hybridization of mRNA in sperm cells, therefore, only can be performed in a pollen tube. Pollen tubes were grown *in vivo* by hand pollinating pistils with freshly collected pollen. After 48 h, a 1-cm segment was taken from the base of the style and was cut into two symmetrical halves. Pollen tubes growing in the hollow stylar canal were teased out, were fixed, and then were used for *in situ* hybridization.

Expression of Recombinant LGCI Protein in *Escherichia coli* and Antibody Production. The coding region of *LGCI* cDNA was cloned into the bacterial expression vector pQE-30 (Qiagen, Chatsworth, CA). The recombinant plasmid was transformed into *E. coli* strain M15. *LGCI* protein expression was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside. The recombinant protein was purified by using Ni-nitrilotriacetic acid agarose affinity chromatography according to the manufacturer's instruction (Qiagen, Chatsworth, CA). Purified recombinant protein was used to raise polyclonal antibodies in rabbits following the standard protocols (12). Antiserum obtained after the third booster was affinity-purified as described (13).

SDS/PAGE, Immunoblotting, and Immunoprecipitation. Protein samples denatured under reducing conditions by boiling in the presence of SDS sample buffer (25 mM Tris-HCl/2% SDS, 2% DTT/10% glycerol, pH 6.8) were subjected to SDS/PAGE according to the method described by Laemmli (14) and then were blotted onto nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked with 10% milk powder in PBS for 1 h, were washed with PBS, and were incubated with affinity-purified anti-*LGCI* antibody (1:200 dilution in PBS containing 1% BSA) for 3 h at room temperature. After washing twice in PBS containing 0.1% Tween 20 and twice in PBS, the blots were incubated in the anti-rabbit antibody conjugated with horseradish peroxidase (1:2,000 dilution in PBS containing 1% BSA) for 2 h. The blots were developed by using 4-chloro-1-naphthol as color substrate.

Immunoprecipitation was performed according to Harlow and Lane (12). Generative cells were resuspended in cell lysis buffer (10 mM K₂PO₄/2 mM EDTA/150 mM NaCl/0.1 mM phenylmethylsulfonyl fluoride/1% Triton X-100), were incubated for 10 min at 4°C, and were centrifuged for 5 min. The supernatant was incubated with affinity-purified anti-*LGCI* antibody for 2 h at 4°C. Fifty microliters of anti-rabbit IgG agarose beads (Sigma) then were added to the mixture and were incubated for 1 h at 4°C. Immunoprecipitates were washed three times with cell lysis buffer and were resuspended in 30 μl of 2 × SDS-sample buffer. The mixture was boiled for 5 min and was centrifuged briefly to precipitate the beads. The immunoprecipitated proteins were subjected to SDS/PAGE and were visualized by staining with Coomassie brilliant blue R250.

Immunocytochemistry. Isolated generative cells were placed on the coverslips coated with poly-L-lysine and were fixed in cold methanol for 2 min. After washing with PBS, the cells were incubated with either anti-*LGCI* antibody or pre-immune serum diluted in PBS containing 1% BSA for 6–8 h in a moist chamber at 4°C. Fluorescein isothiocyanate-labeled secondary antibody was used for the detection of antibody binding. The specificity of the antibodies was confirmed by using recombinant *LGCI* protein as a competitor for antibody binding sites.

RESULTS AND DISCUSSION

Molecular Analysis of the *LGCI* cDNA Clone. Pollen is a two-celled structure in which the generative cell occupies a very small portion of the volume. So far, all of the genes isolated from pollen cDNA libraries have been shown to be specific to the larger vegetative cell (15, 16). To maximize the likelihood of obtaining genes specifically expressed in the generative cell, we constructed a PCR-based cDNA library of generative cells isolated from lily (*L. longiflorum*) pollen. Inserts of cDNA clones picked randomly from the library were used as probes for differential hybridization to RNA from generative cells, pollen, pistil, petal, ovary, leaf, and stem. One cDNA clone, *LGCI*, that showed strong hybridization to RNA of generative cells, weak hybridization to RNA of pollen (containing generative cells), and no detectable hybridization to RNA of other tissues was considered as a putative generative cell-specific clone.

LGCI contains a cDNA insert of 618 bp [nucleotide sequence deposited in the GenBank database (accession no. AF110779)] encoding a predicted gene product of 128 amino acids with a calculated molecular mass of 13.8 kDa and a pI of 5.33. Analysis of the deduced protein sequence revealed an N-glycosylation motif Asn-X-Ser at the C-terminal and a highly hydrophobic region at the N-terminal end. Secondary structure prediction according to Chou and Fasman (7) showed that the hydrophobic region corresponds to an α-helix, indicating that this region may function as a transmembrane anchor and that *LGCI* protein might be associated with the plasma membrane.

Cell-Specific Expression of *LGCI*. The expression pattern of *LGCI* was investigated by using RNA gel blot analysis. *LGCI* hybridized to a transcript of 0.6 kilobase, which is present at a high level in generative cells isolated from mature pollen (Fig. 1A). A faint signal was visible in pollen containing generative cells whereas no hybridization signal was detected in leaf, stem, petal, pistil, and ovule.

We further examined the tissue specificity of *LGCI* by using the more sensitive RT-PCR analysis. RT-PCR amplifications were performed with controlled amounts of RNA from various tissues and two gene-specific primers that amplified a 0.3-kilobase portion of the coding region. A PCR product of the expected size (0.3 kilobases) was obtained in generative cells and pollen but not in any other parts of the plant tested,

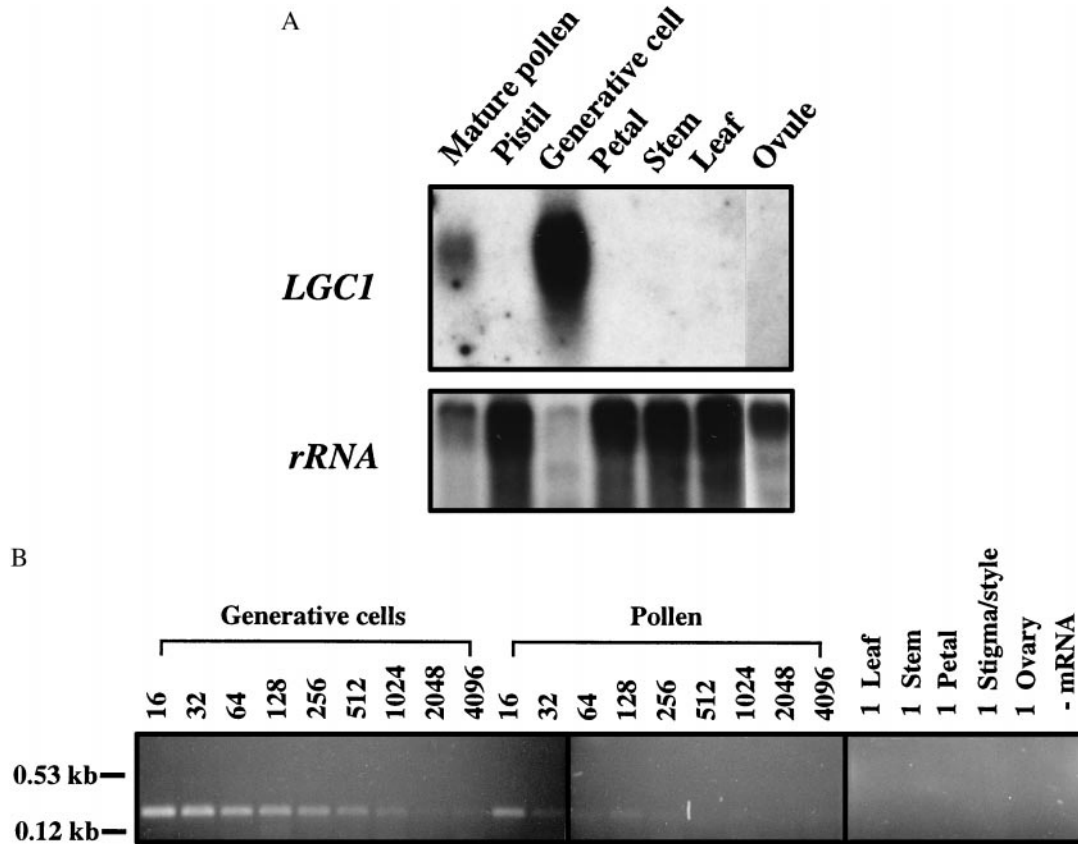


FIG. 1. Expression of *LGCI* mRNA in different tissues of lily. (A) RNA gel blot of the indicated tissues probed with ^{32}P -labeled *LGCI* probe. The blot was reprobed with lily rRNA to verify the relative amount of RNA in each lane. (B) RT-PCR of different tissues as indicated. Pollen mRNA includes contributions of both generative cell and vegetative cell. Numbers 16, 32, and 64 represent 1/16, 1/32, and 1/64, etc., for mRNA input in each lane, respectively. Molecular sizes are indicated on the left.

including vegetative tissues such as leaf and stem as well as reproductive tissues such as petal, female stigma/style, and ovary (Fig. 1B). Based on the signal intensity, we estimated that ≈ 20 -fold more PCR product was obtained when generative cell mRNA was used as compared with pollen mRNA.

The results from RT-PCR confirmed the data obtained by RNA gel blot analysis. In both cases, *LGCI* transcription product was only detectable in the male gametophyte, with much higher levels of expression in the generative cell as compared with that in the entire pollen. We considered that the *LGCI* mRNA detected in pollen owes its origin to the generative cell that constitutes a small portion of the pollen grain.

***In Situ* Localization of *LGCI* Transcripts in Generative and Sperm Cells.** We further analyzed the differential expression of *LGCI* in the vegetative and the generative cell by *in situ* hybridization. The results clearly showed that *LGCI* mRNA is confined to the generative cell but is not present in the vegetative cell of mature pollen (Fig. 2). The strong hybridization signal detected in the cytoplasm of the generative cell appeared as a distinct spindle-shaped form in the pollen grain.

The generative cell is the product of a highly asymmetrical division. It is possible that mRNA present in the generative cell may originate from differential RNA localization and partitioning before generative cell formation, as known during *Drosophila* and *Fucus* embryogenesis (10, 17). To answer this question, the temporal expression pattern of *LGCI* during the process of male gametogenesis was analyzed. We monitored accumulation of *LGCI* mRNA before and after the formation of the generative cell by *in situ* hybridization. *LGCI* mRNA was not detected at any of three stages before microspore division, including microsporocyte,

tetrad, and unicellular microspore stages (data not shown). We further examined six different stages after the formation of generative cells. At the earliest stage, the newly formed generative cell is attached at one pole of pollen with the vegetative nucleus located in its vicinity (Fig. 3A and F). As development progresses, the generative cell starts to detach from the intine (inner cell wall of pollen) while the vegetative nucleus moves toward the center of the pollen (Fig. 3B and G). No detectable signal was observed in either these two early developmental stages (Fig. 3A and B). Concomitant with the rapid size expansion of the pollen, the generative cell detaches completely from the intine and is suspended freely within the vegetative cell cytoplasm. Its shape becomes elongated, with a large nucleus in the center and most of the cytoplasm at both ends of the cell (Fig. 3C and H).

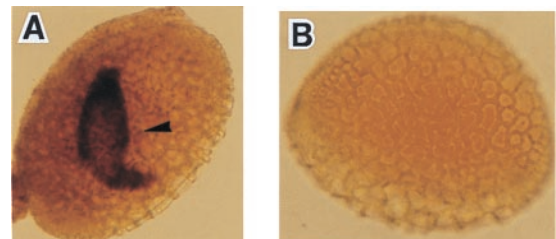


FIG. 2. *In situ* hybridization of *LGCI* mRNA to whole-mounted lily pollen. Dark staining in the generative cell (arrowhead) represents hybridization signal detected by using an alkaline phosphatase conjugated anti-DIG antibody. The outer wall of pollen, exine, appears as a sculptured pattern. (A) Pollen probed with a DIG-UTP-labeled *LGCI* antisense riboprobe. (B) Control pollen probed with a sense riboprobe.

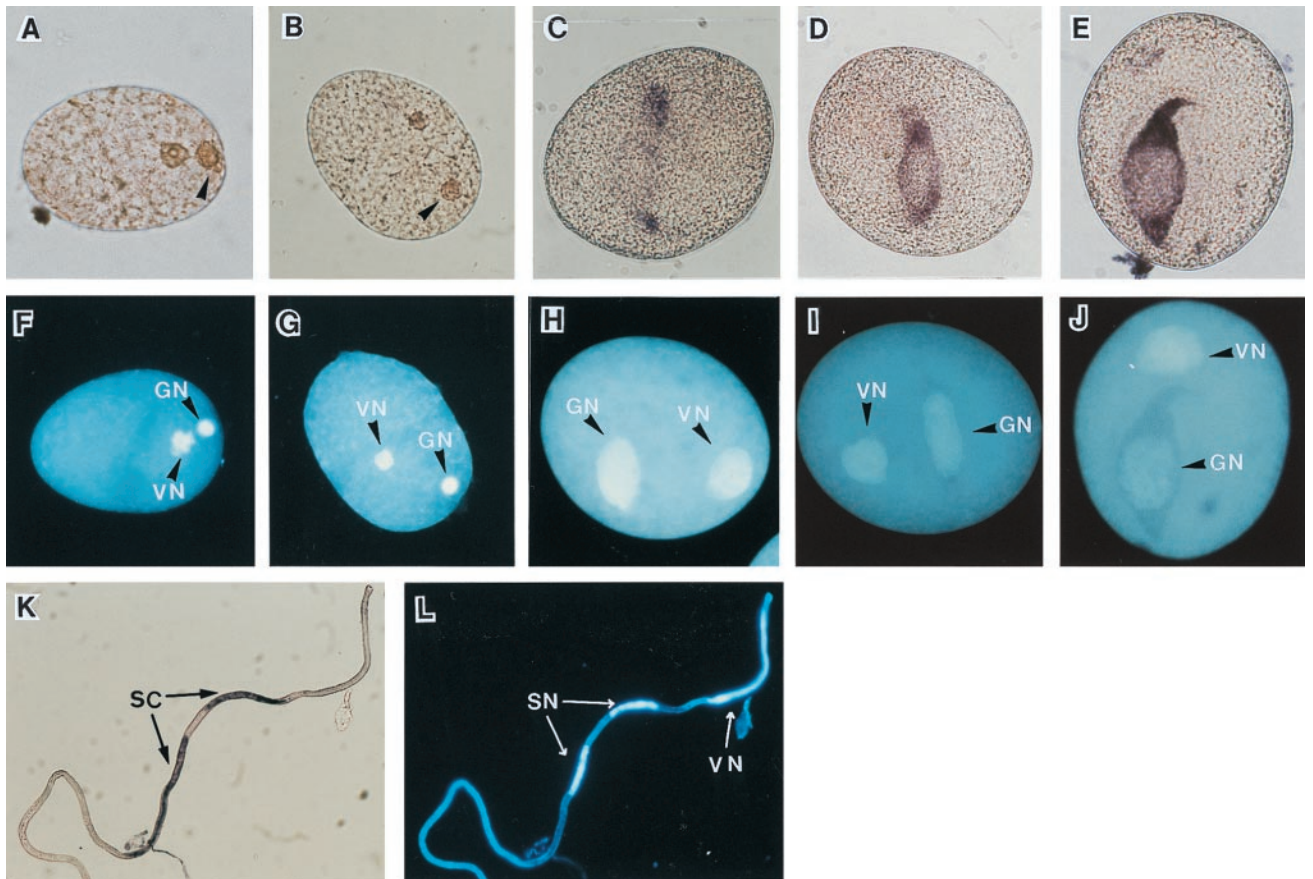


FIG. 3. *In situ* hybridization of *LGC1* mRNA to whole-mounted lily pollen at different developmental stages. For better resolution, protoplasts of developing pollen were released from the sculptured exine. Developing pollen (A–E) and pollen tube (K) were probed with a DIG-UTP-labeled riboprobe and then were counterstained with 4', 6'-diamidino-2-phenylindole to visualize the vegetative and generative nuclei within the pollen (F–J) and to visualize sperm nuclei in the pollen tube (L). Arrowheads indicate the generative cell at early developmental stages. GN, generative nucleus; VN, vegetative nucleus; SC, sperm cell; SN, sperm nucleus.

At this stage, a weak signal was detected at both ends of the generative cell, indicating the initiation of *LGC1* mRNA transcription (Fig. 3C). As development continues, the generative cell becomes spindle-shaped (Fig. 3D and I), and accumulation of *LGC1* mRNA in the generative cell becomes more evident (Fig. 3D). At the time of pollen maturity, a very high level of *LGC1* mRNA was observed in the

generative cell (Figs. 2A and 3E and J). After pollination, pollen germination occurs on the female stigma, and pollen tubes grow inside the female stylar tissue. During this process, the generative cell moves into the pollen tube and undergoes a mitotic division producing two male gametes, the sperm cells (Fig. 3K and L). *LGC1* mRNA was clearly detectable in the two sperm cells inside the pollen tubes (Fig.

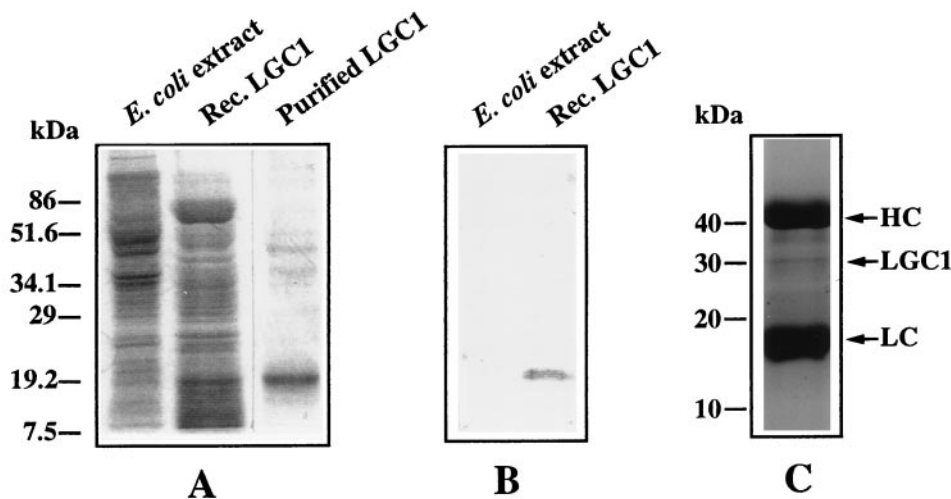


FIG. 4. Immunoblot analysis of *LGC1* protein. (A) Coomassie blue-stained SDS/PAGE gel showing the recombinant *LGC1* protein. (B) Immunoblot probed with anti-*LGC1* antiserum showing specific binding of antibody to *LGC1* protein. (C) *LGC1* protein immunoprecipitated from total protein extract of generative cells. Two strong bands represent the heavy chain (HC) and light chain (LC) of Ig, respectively.

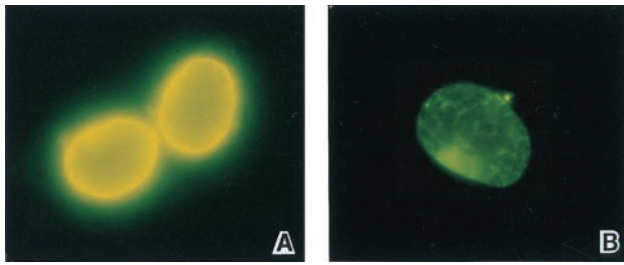


FIG. 5. Localization of LGC1 protein at the surface of the generative cell membrane. Generative cells were incubated with either purified anti-LGC1 polyclonal antibody (A) or preimmune serum (B). Binding of anti-LGC1 antibody was detected by using an anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody.

3K). No hybridization signal was detected in the vegetative cell at any stage of pollen development.

If the generative cell-specific presence of *LGCI* transcript is the result of asymmetric RNA localization and partitioning, we would expect the transcripts to be present in unicellular microspores and early generative cells. Our *in situ* hybridization results indicated the absence of *LGCI* transcript at any developmental stages before the first pollen mitosis. The activation of *LGCI* gene appears to occur at the late bicellular stage when the generative cell is in the free floating state inside the vegetative cell cytoplasm. The expression increases during pollen maturation, reaches the highest level at the mature pollen stage, and remains active in the two sperm cells after division of the generative cell. Based on these results, we conclude that differential localization of *LGCI* transcript in the generative cell is attributable to gene activation in the generative cell.

Characterization of LGC1 Gene Product. To characterize the *LGCI* gene product, we fused the coding region in frame to an affinity tag of six histidines and overexpressed the resulting construct in *E. coli*. The fusion protein had the expected molecular mass of ≈ 19 kDa (Fig. 4A). Polyclonal antibodies raised against the purified protein recognized the recombinant LGC1 polypeptide on immunoblots, confirming the specificity of the antiserum (Fig. 4B). The purified anti-LGC1-antibody then was used to analyze the LGC1 gene product in the plant. A single protein band of ≈ 30 kDa was purified from total protein extract of generative cells by immunoprecipitation (Fig. 4C). The molecular mass of the identified LGC1 protein was higher than that predicted from the deduced amino acid sequence. This discrepancy might be attributable to glycosylation of the protein, which could have occurred at the predicted N-glycosylation site at the C terminus.

To determine the cellular distribution of the *LGCI* gene product, anti-LGC1 antibody was used to localize the protein in the generative cell. From the fluorescence pattern shown in Fig. 5, we conclude that LGC1 polypeptide is present at the surface of the generative cell membrane. The observed localization on the membrane might be attributed to the N-terminal hydrophobic region, which acts as a transmembrane α -helix anchor.

Cell surface localization of LGC1 protein is of considerable interest. Plant male gametic cells lack cell walls, and the first

contact between sperm and egg cells during the process of fertilization occurs at plasma membranes. Recently, an *in vitro* system for adhesion and fusion of maize gametes has been reported (18). Various combinations including sperm-sperm, sperm-egg, and sperm-mesophyll protoplasts were allowed to fuse to test the specificity of fusion. Fusion was found to be mainly restricted to sperm-egg pairs. This result suggests the presence of specific and potentially complimentary fusogenic determinants on the gamete membrane surface. In case of mammalian fertilization, several membrane surface proteins involved in sperm-egg adhesion, fusion, and signaling during the events of fertilization have been identified (19, 20). The strict male gametic cell-specificity of *LGCI* gene expression and localization of its gene product on the membrane surface points toward a putative role in sperm-egg fusion events.

In conclusion, identification of a male gamete-specific gene *LGCI* has opened up opportunities for further investigations into molecular mechanisms involved in fertilization of higher plants. Although still speculative in nature, models for sperm-egg recognition and fusion during double fertilization are emerging from experimental studies using *in vitro* systems (18). The availability of the *LGCI* gene should make it possible to introduce marker genes for monitoring the processes of sperm-egg recognition and fusion at the molecular level.

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