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Developmental expression of polyubiquitin genes and distribution of ubiquitinated proteins in generative and sperm cells

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Abstract Polyubiquitin-encoding cDNA clones were isolated from the generative cells of lily (*Lilium longiflorum*) and the sperm cells of *Plumbago zeylanica*. The described genes encode identical amino acid sequences, with no homology outside the coding regions. This gene participates in ubiquitination of proteins, presumably enhancing protein turnover in the germline during male reproductive differentiation. In this paper we show that the gene encoding polyubiquitin is highly up-regulated in both *Lilium* generative cells and one of the *Plumbago* sperm cell types in particular.

Keywords Angiosperm reproduction · Generative cells · Polyubiquitin · Sperm cells · Ubiquitin

Introduction

Ubiquitin is a highly conserved 76-amino acid protein found in all eukaryotic cells. Ubiquitin is found both free and covalently attached to various nuclear, cytoplasmic and cell membrane proteins. The attachment of ubiquitin to target proteins is achieved through a multi-step enzymatic pathway, which involves ubiquitin-activating E1 enzymes, ubiquitin-conjugating E2 enzymes, and ligating E3 enzymes. Mono- or polyubiquitination of proteins can lead to protein degradation or modification of protein activity. The ubiquitin system has been implicated in many vital cellular processes including cell-cycle control

(Pagano 1997), protein degradation (Rechsteiner 1987), chromatin structure (Barsoum and Varshavsky 1985), chromatin modification in the context of DNA repair and gene silencing (Koken et al. 1996), and response to stress (Belknap and Garbarino 1996; Bond and Schlesinger 1986; Finley et al. 1987).

Ubiquitin appears to have specialized functions in mammalian gametogenesis (Baarends et al. 1999). Mouse gene knockout studies have shown that inactivation of components of the ubiquitin system leads to impaired gametogenesis (Roest et al. 1996). Yeast mutants heterozygous for a deletion of the polyubiquitin gene show reduced viability of spores, and homozygous mutants are sporulation defective (Finley et al. 1987). Ubiquitination of somatic histones has been implicated in their turnover during spermatogenesis (Baarends et al. 1999). Recently, Sutovsky et al. (1999) have shown that uniparental ubiquitin-tagging of mitochondria during spermatogenesis selectively targets sperm mitochondria for postfertilization degradation by proteasomes and lysosomes of the embryo. Characteristic of both spermatogenesis and sporulation is the compact packaging of chromosomal DNA, in which substitution histones result in drastic changes in the composition and condensation of chromatin. Substitution histones associated with plant spermatogenesis have also been reported (Xu et al. 1999a).

In flowering plants, the process of spermatogenesis is initiated by polar migration of the haploid microspore nucleus, followed by a highly asymmetric division that forms a slender generative cell and a much larger vegetative cell. The vegetative cell expresses housekeeping genes associated with pollen metabolism and the development of the pollen tube, whereas the smaller generative cell expresses a more highly focused suite of genes involved in establishing the male germ lineage. The generative cell divides mitotically in the pollen grain or tube to form the two sperm cells. Both of the sperm cells are then utilized during double fertilization, with a divergence of function and cell fate. One of two sperm cells fertilizes the egg cell, establishing the lineage of the

The lily polyubiquitin nucleotide sequence appears in GenBank under the accession number AF116772.

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embryo, whereas the other sperm fuses with the central cell to produce a nutritive, triploid endosperm, which is a terminal lineage in seed formation.

We are interested in identifying genes that are exclusively expressed or upregulated in generative and sperm cells, especially genes involved in control of the cell-cycle, chromatin modification, cell-cell recognition and fusion events during fertilization. With this goal in mind, we have recently constructed PCR-based *Lilium* generative cell and *Plumbago* sperm cell cDNA libraries, and are currently screening such male gamete-specific genes to establish their function in spermatogenesis and fertilization. Here we describe male germ line-specific ubiquitin gene expression that is highly upregulated in both lily generative cells and in the *Plumbago* sperm cell type known to fuse with the central cell during double fertilization.

Materials and methods

Plant materials and cDNA cloning

Generative cells of *Lilium* and sperm cells of *Plumbago* were isolated using published methods (Xu et al. 1998; Zhang et al. 1998), and PCR-based libraries were constructed as described earlier (Xu et al. 1999a). The cDNA libraries were subjected to differential screening using standard protocols. cDNA clones showing preferential or specific hybridization to generative/sperm cell mRNAs were analyzed further.

DNA sequencing was performed on both strands by the dideoxy chain-termination method using an ABI 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 Center for Biotechnology Information.

RNA gel blot and RT-PCR analysis

Total RNA was isolated from generative cells and various tissues using the SNAP RNA purification kit (Invitrogen). Ten micrograms of total RNA were separated on a 1% agarose gel containing formaldehyde, transferred to Hybond N+ nylon membrane (Amersham) and probed with ³²P-labeled *LG52* cDNA insert. Hybridization was performed in 50% deionized formamide, 2×SSPE buffer (1×SSPE is 0.15 M NaCl, 0.01 M NaH₂PO₄, and 1 mM EDTA, pH 7.4), 1% PEG, 0.5% BLOTTO, 7% SDS and 0.5 mg/ml denatured salmon sperm DNA at 42°C overnight. The blots were washed with 2×SSC buffer (1×SSC is 0.15 M NaCl and 15 mM sodium citrate, pH 7.0), 0.1% SDS at room temperature for 15 min and with 0.2×SSC, 0.1% SDS at 65°C for 15 min, followed by a brief wash in 0.2×SSC. The blots were re-probed with lily ribosomal RNA to verify the amount of RNA loaded in each lane. RT-PCR analysis was performed using Access RT-PCR system from Promega.

In situ hybridization

Non-radioactive in situ hybridization was performed on paraffin-embedded, dewaxed sections of *Plumbago* pollen. Both sense and antisense riboprobes labeled with DIG-UTP were generated from linearized DNA templates. Hybridization signal was detected with an alkaline phosphatase conjugated anti-DIG antibody using a DIG nucleic acid detection kit (Boehringer Mannheim).

SDS-PAGE and immunoblotting

Protein samples denatured under reducing conditions by boiling in the presence of SDS sample buffer (25 mM Tris-HCl, 2% SDS, 2% dithiothreitol and 10% glycerol, pH 6.8) were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (1970) and then blotted onto nitrocellulose membranes (Schleicher & Schuell, Germany). Membranes were blocked with 10% milk powder in PBS for 1 h, washed with PBS and incubated with polyclonal antibody (Sigma) to ubiquitin (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 phosphatase (1:2,000 dilution in PBS containing 1% BSA) for 2 h. The blots were developed using CDP-star chemiluminescence substrate. For controls, duplicate blots were processed in the absence of primary antibody. In this case, no non-specific binding of phosphatase-conjugated secondary antibodies was detected.

Results and discussion

Two polyubiquitin genes were cloned in male germ cells of lily and *Plumbago*. The lily generative cell cDNA library clone is *LG52* – a 0.7-kb clone containing a 459 bp open reading frame encoding two ubiquitin units. The N-terminal unit contains 76 amino acids, with a sequence identical to that encoded by *Arabidopsis* and rice ubiquitin genes. However, no homology was found outside the coding region. Using cross hybridization with lily generative cell mRNA to screen sperm-expressed genes in *Plumbago*, we isolated a clone that encodes a polyubiquitin gene with three ubiquitin repeats. Sequence comparison of lily and *Plumbago* clones showed that the homology between the two clones is confined to the polypeptide-coding region for ubiquitin. Although the amino acid sequences of ubiquitin repeats are identical in both clones, the nucleotide sequences are not as highly conserved, displaying only 81% identity.

To determine the specificity of ubiquitin gene expression in lily generative cells, an RNA gel blot was prepared using RNA from various parts of plants including generative cells, whole pollen, leaf, stem and petal. A strong hybridization signal was detected in generative cells and a weak hybridization signal was detected in the cytoplasm of mature pollen (Fig. 1). No hybridization was detected with mRNA from other floral and vegetative organs. The temporal expression pattern of this gene was analyzed using RNA from five different stages of pollen development (microsporocyte, early microspore, late microspore, immature pollen and mature pollen grain). These data indicate that the gene corresponding to *LG52* cDNA is transcribed in the generative cell nucleus.

Ubiquitinated proteins in lily pollen were detected using anti-ubiquitin monoclonal antibody blots at different developmental stages, including developing microspores, early and late bicellular pollen, mature pollen and pollen tubes. The immunoblots showed a number of immunoreactive bands corresponding to the ubiquitinated proteins, including a 9.5-kDa band corresponding to ubiquitin

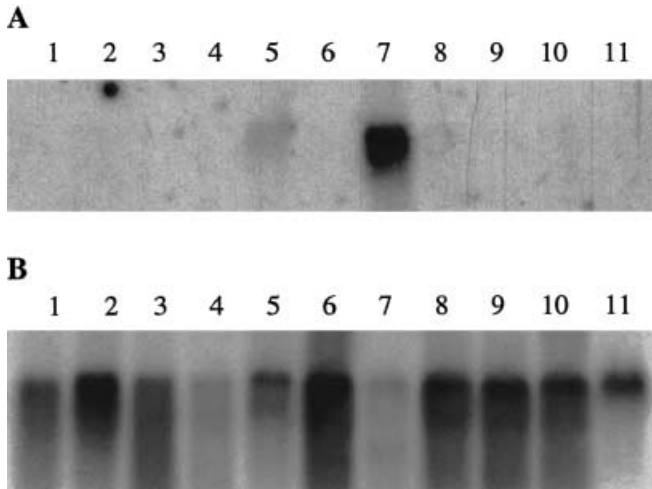


Fig. 1A, B RNA gel blot analysis of lily generative cell poly-ubiquitin gene *LG52* expression. Total RNA was isolated from various tissues and selected stages in the formation of pollen as indicated. Approximately 10 μ g of each RNA was separated on 1.2% denaturing formaldehyde-agarose gels and transferred to Hybond N+ nylon membranes. The membranes were hybridized with a 32 p-labeled *LG52* cDNA insert (A). The blots were washed in 0.2 \times SSC, 0.1% SDS at 65°C for high stringency (Xu et al. 1999b). The blots were re-probed with lily ribosomal RNA to verify equal RNA loading (B). Lanes: 1 microsporocyte, 2 early microspore, 3 late microspore, 4 immature pollen grain, 5 mature pollen grain, 6 pistil, 7 GC (generative cell), 8 petal, 9 stem, 10 leaf, 11 ovule

monomer (Fig. 2), which is close to the molecular mass described for monomeric ubiquitin (8.6 kDa). The pattern of ubiquitinated proteins remained stable from the microspore stage to late pollen development. At maturity, however, there was a general decrease in both the level of ubiquitinated proteins and ubiquitin monomer. In mature pollen, a 63-kDa ubiquitinated protein band appeared that was strongly represented in the ubiquitinated protein pattern of isolated generative cells. These data indicate that the 63-kDa protein band observed in pollen protein extracts may be restricted to the generative cell. In addition to this 63-kDa band, generative cell extracts also showed a number of other anti-ubiquitin immunoreactive bands.

Interestingly, an intense ubiquitinated protein band was observed by Alche et al. (2000) in protein profiles of mature, whole olive pollen. This protein band in olive pollen also was expressed during the final stages of pollen maturation and, similarly, may represent a generative cell-specific ubiquitinated protein.

Due to limited availability of pure material, an RNA gel blot was not feasible for *Plumbago* sperm cells. Therefore, RT-PCR analysis was used to evaluate specificity of ubiquitin expression in sperm cells. Our results (Fig. 3) demonstrate that this polyubiquitin gene is preferentially expressed in mitochondria-rich sperm (S_{vn} type) cells with much higher expression than in plastid-rich sperm (S_{ua}) cells. In situ hybridization experiments on sectioned *Plumbago* pollen also indicates that the hybridization signal occurs in both sperm cells, with

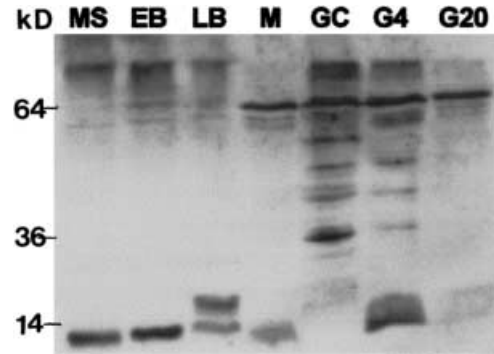


Fig. 2 Immunoblot of free ubiquitin and ubiquitinated proteins at selected stages in the formation of pollen (*MS* microsporocyte, *EB* early bicellular pollen, *LB* late bicellular pollen, *M* mature pollen, *GC* isolated generative cells only, *G4* pollen tubes after 4 h of in vitro germination, *G20* pollen tubes after 20 h of in vitro germination). A 63 kDa GC ubiquitinated protein band becomes apparent at pollen maturity

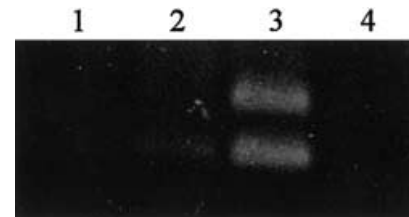


Fig. 3 RT-PCR analysis of ubiquitin gene expression in *Plumbago*, showing a polymer and presumed monomer. Lanes: 1 mature pollen, 2 S_{ua} , 3 S_{vn} , 4 leaf

a higher signal intensity in S_{vn} cells (Fig. 4) and a faint signal in the S_{ua} cells.

Our results suggest an important role for ubiquitin in male gametogenesis of flowering plants. This is the first report of the developmental relationship between expression of the ubiquitin gene and maturation of generative cells of pollen. Previous studies of ubiquitin used exclusively intact pollen as experimental material, and thus are likely to reflect the ubiquitination pattern of the larger vegetative cell. Callis and Bedinger (1994) reported a developmental loss of free and protein-bound ubiquitin during late stages of maize pollen maturation without a loss of ubiquitin mRNA. Based on such observations, Worrall and Twell (1994) concluded that the ubiquitin pathway is not required to effect pollen maturation. Subsequently, Kulikauskas et al. (1995) showed that pollen from most plant families has high levels of ubiquitin monomer. It was also demonstrated that only grasses show drastically reduced levels of ubiquitin monomer in mature pollen. In our studies, we detected an overall decrease in ubiquitin monomer and ubiquitinated proteins in maturing lily pollen. In generative cells, however, ubiquitinated proteins appeared during the final stages of pollen maturation and may be required in the germ lineage. A similar increase in levels of ubiquitinated proteins and the appearance of a number of new ubiquitinated protein bands late in pollen maturation

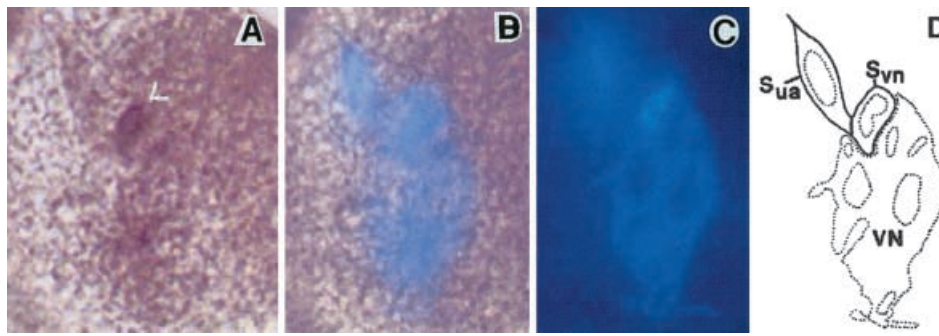


Fig. 4A–D In situ hybridization of polyubiquitin (clone 37). Two preparations are shown in three different lighting regimes: **A** differential interference contrast (DIC), **B** mixed epifluorescence (EF) and DIC, and **C** EF (**D** is a diagram of relative location of cells and nuclei). The anti-sense probe clearly labels the S_{vn} while the S_{ua} shows a faint reaction. The vegetative nucleus (VN) is located below the sperm cells. The sense probe control was not labeled by DIG (not shown)

was also reported in olive pollen (Alche et al. 2000). From our research, we believe that results from mature lily and *Plumbago* pollen suggest relatively high levels of ubiquitin expression in generative and sperm cells. The new ubiquitinated proteins appearing at pollen maturity in generative and sperm cells may thus reflect active ubiquitin-mediated protein turnover and metabolism occurrence in these cells. Recently, we have completed nucleotide sequencing of nearly 1,600 cDNAs from a lily generative cell cDNA library and it is significant to report here that 40 of these cDNAs corresponded to ubiquitin, as compared to 5 H3 histone cDNAs.

The enhanced expression of ubiquitin genes in generative cells and sperm cells suggests that ubiquitin-mediated proteolysis may have a role during the transition of the generative and sperm cells into male, reproductively functioning, cells. Newly ubiquitinated proteins in generative cells may represent male reproduction-triggered turnover of somatic proteins preceding such male germ cell-specific activities as control of terminal cell cycle progression, the expression of substitution histones and DNA repair (Xu et al. 1998). Ubiquitin-mediated protein degradation of existing cyclins is reportedly essential for progression of the cell-cycle, purging the cell of proteins that may inhibit the cascade of events mediating cellular division (Jentsch and Ulrich 1998). For example, in bicellular pollen, generative cells are arrested at the G2/M phase at anthesis, and only complete division upon entry into the pollen tube. Thus, their further progress in the cell cycle may be reflected by high ubiquitination of existing cyclins. In *Drosophila* and *Xenopus*, the proteasome contains ubiquitin subunits that are highly expressed specifically in testis. Male gametic cells of higher plants undergo male gametic cell-specific H2A and H3 histone substitutions (Xu et al. 1999a) presumably relating to chromatin condensation (Thiebaud and Ruch 1978) and their replacement is likely upregulated by ubiquitin. It is interesting to note that the gene encoding the *LG52* ubiquitin gene is not transcribed during pollen

mitosis 1. Thus, the *LG52* expression pattern is similar to genes encoding male gametic cell-specific H2A and H3 histone forms.

In situ hybridization and RT-PCR suggest that one of the two sperm cells, the S_{vn} , which typically contains abundant small mitochondria, shows higher levels of polyubiquitin expression as compared to the plastid-rich S_{ua} . Although it is unclear that there is a functional parallel, it is interesting to note that mitochondria in mammalian sperm cells are highly ubiquitinated as part of the early zygotic elimination of paternal mitochondria (Sutovsky et al. 1999). Dimorphic sperm cells, in addition to organelle differences, may also have characteristic differences in upstream regulation that affect reproductive function. Prior research has indicated that the S_{ua} fertilizes the egg in over 95% of the zygotes observed (Russell 1985, unpublished additional observations). High levels of polyubiquitin in the S_{vn} could be used to reduce or eliminate expression of egg-targeting determinants and therefore may serve to enhance fusion with the central cell.

The genes encoding ubiquitin are among the most highly conserved genes in eukaryotes, with nearly identical amino acid sequences reported across the widest spectrum of organismal diversity (Varshavsky 1997). High levels of ubiquitin polypeptide gene expression under the control of a male gametic cell specific gene promoter may be required to regulate different ubiquitin-mediated pathways important for flowering plant gametogenesis.

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