Available online at www.sciencedirect.com
SCIENCE DIRECT
Plant Science 170 (2006) 433–440
PLANT SCIENCE
www.elsevier.com/locate/plantsci

Biochemical characterization of a pollen-specific cDNA encoding polygalacturonase in *Lilium longiflorum*

Jin-Yi Chiang, Ssu-Wei Shu, Chyng-Wen Ko, Co-Shine Wang *

Graduate Institute of Agricultural Biotechnology, National Chung Hsing University, Taichung 40227, Taiwan

Received 8 June 2005; received in revised form 10 September 2005; accepted 27 September 2005
Available online 12 October 2005

Abstract

A pollen-specific transcript, designated *LLP-A1.1*, in lily (*Lilium longiflorum* Thunb. cv. Snow Queen) plants was isolated and characterized. The full-length cDNA encodes polygalacturonase (PG) having a sequence of 413 amino acids, a calculated molecular mass of 44 kDa, and a calculated p*I* of 8.1. Assessment of the hydropathy shows that the protein contains a hydrophobic segment at the N-terminus, indicating the presence of a putative signal peptide. The LLP-PG sequence displays significant resemblance to known pollen PG from various species and a group of major allergens including Phl p 13 of timothy grass and Pla a 2 of London planetree. Antiserum was raised against the overexpressed rLLP-PG protein in *E. coli*. Affinity-purified antibodies were prepared from antiserum to investigate the specificity and distribution of the protein during development. Immunoblot analyses of total protein from floral and vegetative organs confirmed that LLP-PG accumulated to detectable levels only in a discrete stage of anther development. It is a heterogeneous glycoprotein, consisting of four major and two minor polypeptides. Premature drying of developing anthers indicated that the lily *LlpPG* gene expression was not induced by desiccation that naturally occurs in pollen prior to anthesis.

Keywords: cDNA; *Lilium longiflorum*; Pollen-specific; Polygalacturonase

1. Introduction

Male gametogenesis is an ideal system for studying development. Although relatively reduced in morphological complexity, pollen does undergo dynamic processes of differentiation and performs several specialized functions. Cytological and biochemical research has revealed that the cytological changes in pollen are accompanied by the synthesis of mRNAs [1–3] and proteins [4–8], indicating a function for these macromolecules during microgametogenesis. Pollen-specific genes from various species have been characterized and, apart from 'housekeeping' genes that are active throughout male gametogenesis, can generally be categorized based on the timing of the expression. Early genes are expressed before the first mitosis and late genes are post-mitotically expressed [9]. The vast majority of pollen-specific cDNA clones that have been isolated belong to the ''late'' genes. Products from these genes may be involved in pollen maturation, germination and tube growth [10,11]. In many species, including maize, tobacco, *Brassica napus*, cotton, willow, and others, cDNA with sequence similarity to polygalacturonase (PG) have been identified and classified as late genes [12–16]. However, *QRT3* in Arabidopsis encodes a PG homolog specifically and transiently expressed in the tapetum during the early microspore stages of pollen development [17].

PGs that act as pectin-degrading enzymes are widely distributed in plants, fungi, and bacteria. They catalyze the degradation of highly polymeric galacturonate, a major component of pectin in plant cell walls, into individual galacturonic acid residues [18]. PGs participate in many stages of plant development that include organ abscission zones, pod and anther dehiscence, microspore/pollen development, pollen tube growth, and symbiosis with rhizobia [17,19,20]. Many PG

Abbreviations: ABA, abscisic acid; BSA, bovine serum albumin; Con A-HRP, concanavalin A conjugated with horseradish peroxidase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; MOPS, 3-[*N*-Morpholino]propanesulfonic acid; PBS, phosphate buffer saline; PG, polygalacturonase; PMSF, phenylmethylsulfonyl fluoride; RACE, rapid amplification of cDNA end; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBST, Tris-buffer saline/Tween; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis

* Corresponding author. Tel.: +886 4 2284 0328x771; fax: +886 4 2285 3527.
E-mail address: cswang2@dragon.nchu.edu.tw (C.-S. Wang).

0168-9452/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.plantsci.2005.09.010

genes from various species have been identified and characterized [19,21], however, only a few papers have characterized the PG proteins [12,22]. Herein, we have used biochemical and immunological analyses to characterize LLP-PG encoded by *LLP-A1.1*. The LLP-PG is a heterogeneous, pollen-specific glycoprotein. The protein accumulates only at the stage of pollen maturation, but its accumulation is not induced by desiccation that naturally occurs prior to anthesis.

2. Materials and methods

2.1. Plant materials and treatments

Plants of lily (*Lilium longiflorum* Thunb. cv. Snow Queen) were grown in the field. Buds ranging from 10 to 170 mm were dissected to isolate anthers which varied in length from 7 to 23 mm. Meiosis occurred in the pollen mother cells at bud size around 20–25 mm, resulting in the formation of tetrads. Afterward microspore mitosis was complete at bud size around 65–70 mm and pollen subsequently entered the maturation phase of development. The anther was separated from the filament in buds longer than 25 mm; otherwise the two organs were combined. Pollen was manually collected 1 or 2 days after anthesis. The first three arrays of young leaves around buds, entire roots (approximately 8 cm from the apex), and stems were collected and frozen immediately in liquid nitrogen. Material was stored at −80 °C until use.

2.2. DNA sequencing

A λZapII cDNA library was constructed from poly(A)$^+$ RNA isolated from mature pollen [2]. The cDNA insert was excised in vivo from recombinant λZapII phage and recircularized to form recombinant pBluescript SK(-) phagemid according to the manufacturer's instructions (Stratagene). Complete DNA sequence from both strands of the cloned inserts was obtained using an ABI 3730XL DNA analyzer (Foster City, CA, USA) done by Mission Biotech Co. Ltd. (Taipei City, Taiwan). 5′- and 3′-RACE was done according to the user manual of SMART^{TM} RACE cDNA amplification kit (CLONTECH Laboratories, Inc., CA, USA). Sequence comparison was achieved using the Cluster method in DNA Star Program (DNASTAR Inc., Madison, USA) and the homology search was done with the BLAST program [23].

2.3. Overexpression and purification of LLP-A1.1 protein in E. coli

Polymerase chain reaction (PCR) was used to amplify *LLP-A1.1* cDNA. This was carried out using the pLLP-A1.1 cDNA as a template, with two gene-specific primers. The two gene-specific primers, one of which is LLP-A1.1-for. (5′-CCGGAATTCCATATGGCTTTTAGGGGCCCC-3′) containing an *Eco*RI site followed by *Nde*I, another is LLP-A1.1-rev. (5′-CCGCTCGAGATGTACTTGAGGGCTGATATC-3′) containing *Xho*I. Thus, a 888 bp *LLP-A1.1* fragment containing a partial sequence of LLP-PG starting from 78 to 373 residues was therefore cloned into the *Eco*RI/*Xho*I-cut pET32a expression vector (Novagen) to generate a recombinant *LLP-A1.1* expression plasmid designated pET32a-LLP-A1.1. The resultant construct was transformed into *E. coli* strain DH10B. Plasmid DNA was isolated by Plasmid Miniprep Purification Kit (GeneMark Technology Co. Ltd., Tainan, Taiwan). The purified plasmid was then transformed into *E. coli* strain BL21 (DE3). The bacterial cells were grown on LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract, and 170 mM NaCl, pH 7.0) containing ampicillin (50 μg ml^{-1}) at 37 °C until an OD_{600} between 0.6 and 0.8 was reached. At this point, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and cells were incubated for additional 4 h. Samples of cells before and after induction were harvested by centrifugation at 3000 × *g* for 10 min. Total cell pellets were resuspended in 200 μl of resolubilization solution [100 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Tris–HCl, pH 7.4], sonicated and centrifuged at 16,000 × *g* for 5 min. The fraction of supernatant was recentrifuged, precipitated by acetone and resuspended in the resolubilization solution. On the other hand, the fraction of pellet was washed twice with resolubilization solution and finally dissolved in the inclusion buffer [62 mM Tris base, 0.1% (w/v) SDS, and 5% (v/v) ß-mercaptoethanol]. Protein from the two fractions was electrophoresed by SDS-PAGE and stained with Coomassie blue. The fusion protein was found in the pellet fraction.

2.4. RNA blot

Total RNA was extracted from developing anthers using the Ultraspec RNA^{TM} isolation system (Biotecx Laboratories Inc., Houston, USA). RNA samples were electrophoresed in 1.0% formaldehyde-MOPS gels using standard procedures [24] and transferred onto nylon membranes (Micron Separation Inc.). Membranes with immobilized RNA were prehybridized for 4 h at 42 °C in medium containing 5 × SSC (0.75 M NaCl, 0.075 M sodium citrate, pH 7.0), 0.1% polyvinylpyrrolidone, 0.1% ficoll, 20 mM sodium phosphate, pH 6.5, 0.1% (w/v) SDS, 1% glycine, 50% formamide and 150 μg ml^{-1} of denatured salmon sperm DNA. For hybridization, the prehybridization solution was removed and replaced with hybridization buffer that contained the same components as the prehybridization buffer except 1% glycine, and denatured salmon sperm DNA (100 μg ml^{-1}) and random-primed ^{32}P-labeled probe (specification 8.0 × 108 cpm μg^{-1}). Hybridization was carried out at 42 °C overnight with constant agitation. Membranes were washed at 42 °C twice in 2 × SSC, 0.1% (w/v) SDS for 20 min followed by twice in 0.1× SSC, 0.1% (w/v) SDS at 60 °C for 20 min. The membrane was exposed to X-ray films (Konica AX) using 1 or 2 intensifying screens (DuPont).

2.5. Preparation, electrophoresis and immunoblotting

The phenol extraction method was used to extract total protein from various organs of lily plants, from anthers of