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Full Length Article

Purification, Characterization and Localization of Reversibly Glycosylated Polypeptide 1 from Rape (*Brassica napus* L.) Bee Pollen

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Abstract

Rape (*Brassica napus* L.) bee pollen with plenty of nutrients is the male reproductive spores of rape collected by honeybee. Reversibly glycosylated polypeptide (RGP), as an enzyme capable of self-glycosylated, is crucial to synthesis of cell wall polysaccharides and essential to pollen mitosis and microspore development. However, previous studies have little information about RGP in the bee pollen. In present study, protein from rape pollen was precipitated by ammonium sulphate, extracted and purified by ion exchange and column chromatography. The identification and homology analysis of RGP were carried out by MALDI-TOF-MS and biological software MASCOT with NCBI-nr database. Subsequently the results were verified by MALDI-TOF-MS/MS. Meanwhile, the subcellular localization of RGP pollen anthers in rape were studied by immunohistochemistry. This purified protein belonged to the class 1 reversible glycosylated polypeptide (RGP1). Protein electrophoresis results showed that RGP1 was composed of one subunit with the apparent molecular mass about 40.6 kDa. RGP1 existed in the cytoplasm, the extine of the anther cell and outside of the anther cell. Ours results not only enrich the basic research in RGP related fields, but also provide a theoretical basis and data support for further revealing the role of RGP in the development and formation of rape pollen. © 2019 Friends Science Publishers

Keywords: Characterization; Purification; Rape pollen; Reversible glycosylated polypeptide

Introduction

Plant cell walls, as one of the most abundant resources on earth (Welner *et al.*, 2017), are recalcitrant copolymeric structures mainly comprising polysaccharides (pectin, hemicelluloses, cellulose) (Agger *et al.*, 2014; Beatrice *et al.*, 2017), glycoproteins (Bellincampi *et al.*, 2014) and lignin (Altartouri and Geitmann, 2015). In addition to cellulose and callose, plant cell wall polysaccharides are synthesized in the Golgi apparatus and then transferred via exocytosis to the cell wall through secretory vesicles (Dhugga *et al.*, 1997) where they inserted into cellulose microfibrils in the plasma membrane formed and deposited directly into the cell wall (Sandhu *et al.*, 2009). Synthesis of polysaccharides requires complex, well-coordinated biosynthetic machinery included a specialized procedure called autocatalytic glycosyltransferases (Held *et al.*, 2015) which were known as the first step of plant polysaccharide biosynthesis during a long time (Moreno *et al.*, 1987).

In the search of enzyme capable of self-glycosylated, a multigene family of reversibly glycosylated polypeptide (RGP) belonging to self-glycosylating highly (De pino *et al.*, 2012) and plant-specific proteins (Zavaliev *et al.*, 2009) was discovered in the early 1990s (Dhugga *et al.*, 1991, 1997; Delgado *et al.*, 1998; Bocca *et al.*, 1999). Subsequently, several RGP genes have also been isolated from various plant species including cotton (Zhao and Liu, 2001), bread wheat (Langeveld *et al.*, 2002), rice (Langeveld *et al.*, 2002), potato (Testasecca *et al.*, 2004), maize (Sagi *et al.*, 2005) and tomato (Selth *et al.*, 2006).

The presence of the RGP in both monocots and dicots rather than non-plant system suggests that it may have special functions for plant (Wu *et al.*, 2005). The homologous sequence comparison of RGP cDNA clones in wheat and rice showed that there exist two types of RGP proteins termed RGP1 and RGP2 (Langeveld *et al.*, 2002). Functional study indicated that class 1 RGPs may play a unique role in the biosynthesis of polysaccharides and cell wall synthesis, while class 2 RGPs may be necessary for starch biosynthesis (Langeveld *et al.*, 2002). Electron microscopy immunolocalization manifested that RGP is located in the body of Golgi dictyosomes. Moreover, it was reported that RGP 1 from *Arabidopsis thaliana* is a plasma-associated protein transmitted to plasmodesmata through the Golgi

apparatus (Sagi *et al.*, 2005). Dhugga *et al.* (1997), who first discovered the RGP from pea hypocotyls, demonstrated that RGP1 can be glycosylated by radiolabeled UDP-Xyl, UDP-Glc, UDP-Gal and radioactive sugar is removed from the protein when unlabeled UDP-sugar or UDP is added. Substrate specificity and localization jointly support the role for RGP1 in biosynthesis of hemicellulose polysaccharide (Dugard *et al.*, 2016).

Bee pollen, the male reproductive spores of flowering plants collected by honeybee, is usually mixed with added nectar and bee secretions (Dong *et al.*, 2015) and contains rich nutrients such as vitamins, lipids, carbohydrates, proteins, minerals and flavonoids (Ares *et al.*, 2017; De-melo and De almeida-muradian, 2017). As a miniature nutritional reservoir, bee pollen has been used as a "perfect health food" (Zhang *et al.*, 2016). Rape (*Brassica napus* L.) bee pollen possesses plenty of therapeutic and nutritional properties and commonly used as a nutraceutical or traditional medicine (Sun *et al.*, 2017). Previous studies revealed that rape bee pollen exhibited effectively therapeutic benefits including cardioprotective (Lv *et al.*, 2015), anti-allergic (Medeiros *et al.*, 2008), antioxidant (Leblanc *et al.*, 2009) and anti-inflammatory activity (Wagenlehner *et al.*, 2009). The RGPs are needed during pollen mitosis and microspore development by affecting vacuolar integrity and / or cell division (Drakakaki *et al.*, 2006). As far as we know, there is no report about the RGP in the rape pollen. In this paper, we purified a new protein from rape pollen precipitated by ammonium sulfate, purified by ion exchange and gel filtration chromatography, and identified by MALDI-TOF mass spectroscopy was finally proved to be RGP1.

Materials and Methods

Material and Reagents

Rape bee pollen was purchased from Beemaster Honey Industry (Shaanxi, China). Coomassie brilliant blue G250 and bovine serum albumin (BSA) were purchased from Amresco (OH, USA). β-Mercaptoethanol, tris (hydroxymethyl) aminomethane (Tris), TEMED, sodium dodecyl sulfate (SDS)were obtained from solarbio Science & Technology Co. (Beijing, China). All other reagents used in the experiment were of analytical grade or purer.

Preparation of Crude Protein

The method of breaking the pollen wall is based on Hu *et al*. (2005), Yang and Dang (2010) with slight modifications. Rape pollen (80 g) was grinded before frozen at -20°C for 24 h. Pollen powder was dissolved by addition of hot water (85°C) at the scale of 1:4 (w/v). Then treated under ultrasonic condition for 15 min and stored at 40°C for 6-8 h.

Crude proteins were precipitated by ammonium sulfate. Phosphate buffer saline (PBS 0.1 M, pH 7.4) was added 450 mL to above mentioned solution. Start stirring, extracted for 8 h and incubated for 1 h at 4°C, subsequently, centrifuging at 4800 r/min for 10 min to remove the insoluble materials. Then the supernatant was collected and fractionated by 5 fractions according to stepwise salting-out of saturated ammonium sulfate (0-20% 20-40%, 40-60%, 60-80% and 80-100%). Each faction was dissolved in PBS buffer (0.1 M, pH 7.4) and dialyzed for 24 h to completely remove ammonium sulfate and stored at -20°C for further use.

RGP1 Purification

The dialyzed crude protein from the 20-40% saturation fraction of the rape bee pollen was further purified by IEC in a column DEAE-Sepharose Fast Flow previously washed by distilled water for about 1 h and equilibrated in PBS buffer (0.1 M, pH 7.4) for about 2 h. After protein sample was loaded, the ion exchange column was washed by the same buffer and then eluted by a linear gradient of 0-2 M NaCl dissolved in the above buffer at a flow rate of 2 mL/min. Fractions containing RGP1 were collected and concentrated, and finally loaded to Sepharose CL-6B gel filtration chromatography (GFC) washed before by distilled water for about 2 h and equilibrated in 0.1 M PBS (pH 7.4) for about 2 h. After protein was loaded, the gel column was eluted by PBS buffer (pH 7.4, 0.15 M NaCl) at a flow rate of 2 mL/min. Fractions containing RGP1 were pooled and concentrated. The protein concentration was determined in accordance with the Bradford method, with BSA as a standard (Bradrord, 1976).

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to check the molecular weight and purity of the purified protein. Proteins obtained were analyzed by SDS-PAGE (1.5 mm thickness) according to the method of Leammli (1970) with 5% stacking gel and 12% resolving gel. Protein samples were suspended in the sample buffer at the volume ratio of 4:1 and the solution was heated in boiling water for 5 min prior to loading. Gels were stained with Coomassie brilliant blue R-250 for 30 min and then destained by destaining solution including 20% ethanol and 7% glacial acetic acid until the band on the gel was clear. 1 mL sample buffer contains 0.1 mL anhydrous glycerol, 1 mL 0.05 M Tris-HCl (pH 6.8), 10 mg SDS, 0.2 mg bromophenol blue, and 0.01 M β-mercaptoethanol.

Peptide Mass Fingerprinting (PMF)

Prior to analysis, protein gel bands were excised from the gel and soaked in 50-100 μL of a 25 m*M* NH_4HCO_3 solution containing 50% acetonitrile (ACN). After oscillating 20 min in the oscillator, the gel piece was dried in a SpeedVac. To obtain the peptide, 10 μL trypsin solution (10 mg/mL in 50 m*M* NH_4HCO_3) was added to the tubes of gel piece and incubated overnight (16-18 h). The peptides were recovered from the gel pieces by adding 10-20 μL of extraction

solution of 5% trifluoroacetic acid (TFA) and 50% ACN. The extracted peptide solution was dried to about 4-5 μL and stored at -20ºC for future use. MALDI-TOF-MS and MALDI-TOF-MS/MS were obtained from Bruker ultraflex III TOF/TOF (Bruker Daltonik GmbH, Germany) in reflected positive ion mode using α-cyano-4-hyroxy-cinnamic acid (CHCA) (10 mg/mL in 0.1%TFA /50% ACN) as the matrix. PMFs gained were searched through the SWISS-PROT and NCBI-nr database via the MASCOT search engine (http://www.matrixscience.com), one of which was missing the cleavage site. The peptide amino acid sequence was deduced using the peptide-sequencing program MasSeq.

Subcellular Localization

On LKB-V microtome, ultrathin sections (50-70 nm thick) were cut using a glass knife (Delecoll *et al.*, 1992). BSA (3%) was used to block the sections for 30 min and PBS buffer (0.1 M, pH 7.4) was used to remove the redundant BSA. Anti-RGP1 rabbit antiserum was added and after incubated overnight at 4°C. PBS was used to remove the unbound antiserum three times. Then the samples were incubated with goat anti-rabbit IgG conjugated 15 nm colloidal gold diluted 1:50 in 0.01 M TBST (Tris-HCl buffer containing 0.02% Tween) for 50 min at room temperature. After washing three times with distilled water, the samples were stained with lead citrate and uranyl acetate for 30 s respectively and observed under H-7650 transmission electron microscope.

Results

RGP1 Purification

RGP1 was extracted from rape bee pollen by ammonium sulfate precipitation. The SDS-PAGE indicated there are many kinds of protein, among when the ammonium sulfate saturation is 20-40%, the band is legible and abundant (Fig. 1A). Purification steps were carried out in sequence: The IEC step obtained two main peaks (Fig. 2A). Fractions corresponding to peak 1 were collected, concentrated and subjected to GFC as peak 1 contains the interest protein. The GFC procedure as well revealed two peaks (Fig. 2B). Fractions corresponding to peak 1 were collected and concentrated for SDS-PAGE. The SDS-PAGE result revealed only one band (Fig. 1C) with an apparent molecular weight of approximately 40 kDa, proving that the protein in peak 1 was purified to homogeneity and included only one kind of subunit. The molecular weight showed in the SDS-PAGE result is in agreement with the MASCOT research results (see below), which is 40.6 kDa.

PMFs of the RGP1

MALDI-TOF-MS is an effective tool to execute unknown protein identification in virtue of PMF. A series of process include separation of proteins by gel electrophoresis, cleavage or digestion of proteins using trypsin, mass analysis to determinate the molecular masses or generate peptide fragments, and then comparison with theoretical peptide masses by means of SWISS-PROT and NCBI-nr database via the MASCOT program. With regard to results, there exist

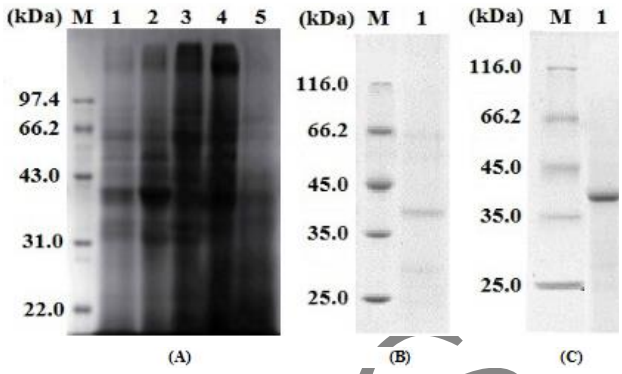

Fig. 1: SDS-PAGE analysis of RGP1 fractions. (A) Lane M, molecular markers; Lane 1-5, precipitated proteins obtained at 0-20%, 20-40%, 40-60%, 60-80%, and 80-100% saturation with ammonium sulfate; (B) Lane M, protein markers; Lane 1, fraction of peak 1 from DEAE F. F. column; (C) Lane M, protein markers; Lane 1, pure RGP1 of peak 1 from Sepharose CL-6B column

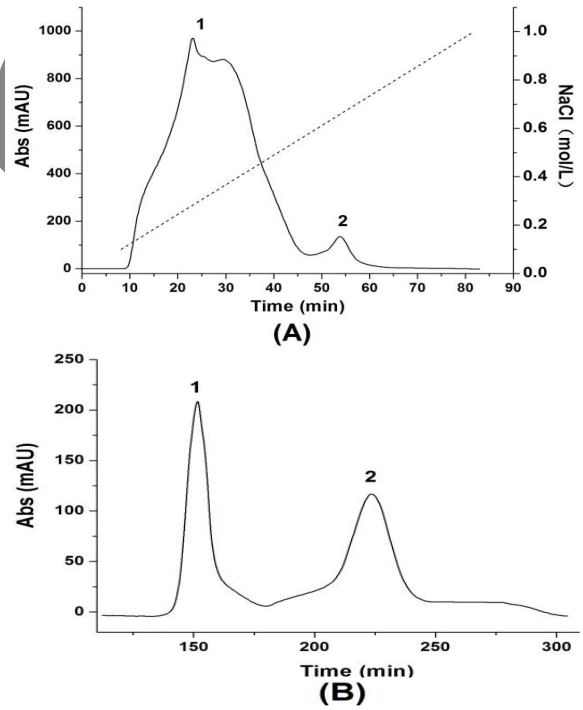

Fig. 2: IEC elution profile of RGP1 on column DEAE-Sepharose Fast Flow (A) and Sepharose CL-6B gel filtration column (B). Proteins were monitored by measuring absorbance at 280 nm

several vital information, such as mowse score, sequence coverage, mass values matched. (Thiede *et al.,* 2005). The MALDI-TOF spectrum of peptides indicated the presence of three relative abundance m/z 839.4105, 1786.9795, 3035.7790 peptide ions in tryptic PMF of the RGP1 (Fig. 3A). Subsequently, the peptide mass data for the 40 kDa gel band listed in Table 1 was searched in the protein database NCBI-nr 2012114 containing 2,161,597,2 sequences entries using the MASCOT search program (http://www.matrixscience.com). Protein scores greater than 86 are considered significant ($p < 0.05$) (Table 2). It is found that the score of the purified protein is 264 and the protein is proved to be Reversible Glycosylated Polypeptide 1 (RGP1). Sequences in black are the amino acid sequences of AtRGP1 and in red are RGP1 in this study (Fig. 3A). Sequences shown in red have 60.56% identity with that of the AtRGP1 from the MASCOT program. Our results demonstrated that the protein purified in this study belongs to the class 1 RGPs, therefore we named it RGP1.

To confirm the above conclusion, the three stronger PMF peaks at m/z of 839.4105, 1786.9795 and 3035.779 were analyzed by MALDI-TOF-MS/MS (Fig. 4). Corresponding to AtRGP1, amino acid sequences respectively have perfectly matched with GYPFSLR, AVPEGFDYELYNR and LWL (underlined). The results of MALDI-TOF-MS/MS further confirm the above conclusion that the protein we purified is RGP1.

Table 1: Mass peaks of gel band 40.0 kDa for MASCOT database search

Gel Band 40.0 kDa of RGP1 PMF (m/z)		
839.4105	1745.8890	2138.1958
991.5399	1761.8907	2154.1783
1178.6777	1786.9795	2317.2498
1210.7108	1926.0917	2365.2514
1606.8420	1969.0631	2384.3845
1667.9313	1985.0769	2399.3005
1729.8714	2091.1248	3035.7790

Table 2: MASCOT search parameters for the gel band of 40.0 kDa

Type of search	Peptide mass fingerprint
Enzyme	Trypsin
Mass Values	Monoisotopic peak
Protein Mass	40 kDa
Peptide Mass Tolerance	± 0.3 Da
Fragment Mass Tolerance	± 0.5 Da
Max Missed Cleavages	2
Number of queries	82 for 40 kDa

Subcellular Location

To locate the RGP1 in the anther cell of rape and get to know the function of RGP1 in the development of rape pollen, the method of immunological histological chemistry was used. Anther cell was clearly observed in the samples and immunogold particles were present in the cytoplasm, the extine of the anther cell and outside of the anther cell (Fig. 5).

Discussion

Rape bee pollen is of high nutritional value, containing proteins, nucleic acid, carbohydrates, lipids, vitamins, minerals and physiologically active substances (Fatrcová-Šramková *et al.,* 2013; Wang *et al.,* 2018). Reversibly glycosylated polypeptide (RGP) is highly conserved membrane-peripheral proteins found in monocots and dicots (Kuttiyatveetil and Sanders, 2017). RGP is involved in the metabolism and growth regulation of many species of plants. In addition, it is also closely related to the defense response of plants (Selth *et al.,* 2006), impairment of virus transmission (Zavaliev *et al.,* 2009), and response to abiotic stresses (Hukkanen *et al.,* 2008). Besides, membrane-associated RGP1 who is localized in either Golgi apparatus or cytoplasm can autoglycosylate characterized by testing glycosylation activity with the help of unlabeled UDP-Glc, UDP-Xyl and UDP-Gal (Dhugga *et al.,* 1997; Welner *et al.,* 2017). Previous studies confirmed that it is associated with the synthesis of polysaccharide and the formation of cell wall. We found that the content of RGP is high (approximately 75 μg/g), however, there is little information about the property and biological function in rape bee pollen. In this paper, the RGP1 was isolated, purified, characterized and localized by the analysis of the water-soluble protein in rape bee pollen.

Primarily, the molecular weight of the protein we purified was ~40 kDa, which was in the range of separated from different plant sources reported by other authors. For example, the molecular weight of RGP1 purified from pea by Dhugga *et al.* (1997) and from potato by De Pino *et al.* (2007) was about 40 kDa, while, RGP purified from Zea mays by Sagi *et al.* (2005) and from nasturtium fruit by Falk *et al.* (2000) was 41 kDa.

Subsequently, to obtain more identification information of interest protein, SDS-PAGE gel bands of the RGP1 were subjected to MALDI-TOF mass spectrometry using CHCA as the matrix. Meanwhile, matching the protein in the NCBI-nr database with the MASCOT search engine to identify the protein and further validate the results using MALDI-TOF-MS/MS. The molecular weight of this protein was calculated to be 40604 Da and the isoelectric point was 5.61. Sequence comparison matched with the amino acid sequence of AtRGP1 in NCBI-nr database was 60.56%, which was determined as RGP1.

According to relevant literature, the RGP1 and RGP2 are required during pollen development, either affecting cell division and/or vacuolar integrity (Drakakaki *et al.,* 2006). Saxena and Brown Jr (1999) thought that the RGPs may bind firmly to nucleotid sugars in the cytosol and then transfer them to the Golgi where they deliver the sugars to an acceptor for the polysaccharide biosynthesis. Therefore, to locate the RGP1 in the anther cell of rape, we carried out immunological histological chemistry and confirmed RGP1 situates in cytoplasm at last.

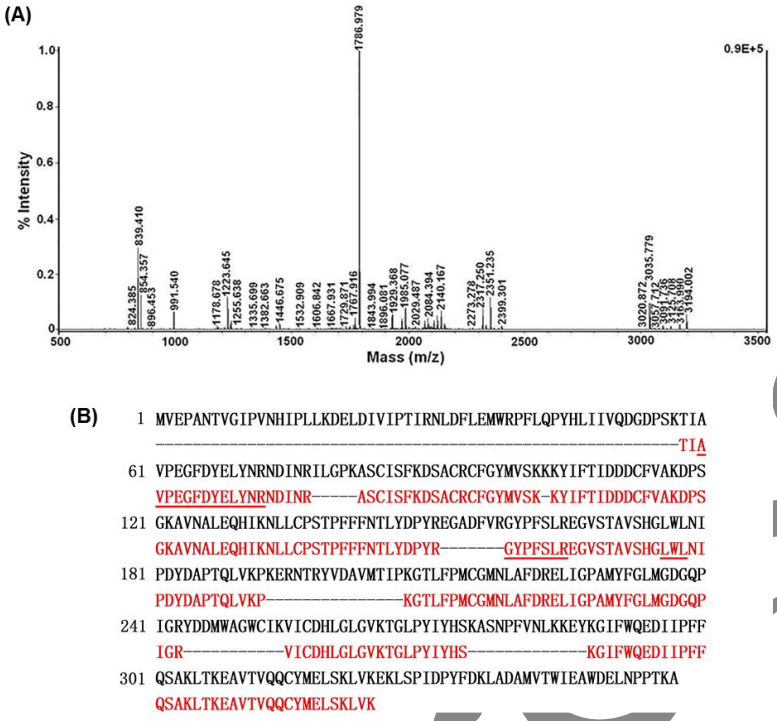

Fig. 3: (A) Tryptic PMF of the 40.0 kDa gel band of RGP1; (B) Amino acid sequence alignment between the peptides of RGP1 and Arabidopsis thaliana RGP1 (AtRGP1). Sequences of the RGP1 purified in this paper matched with MASCOT database research are exhibited in red blow the sequences of AtRGP1. Identified amino acid sequences acquired by MALDI-TOF-MS/MS are underlined

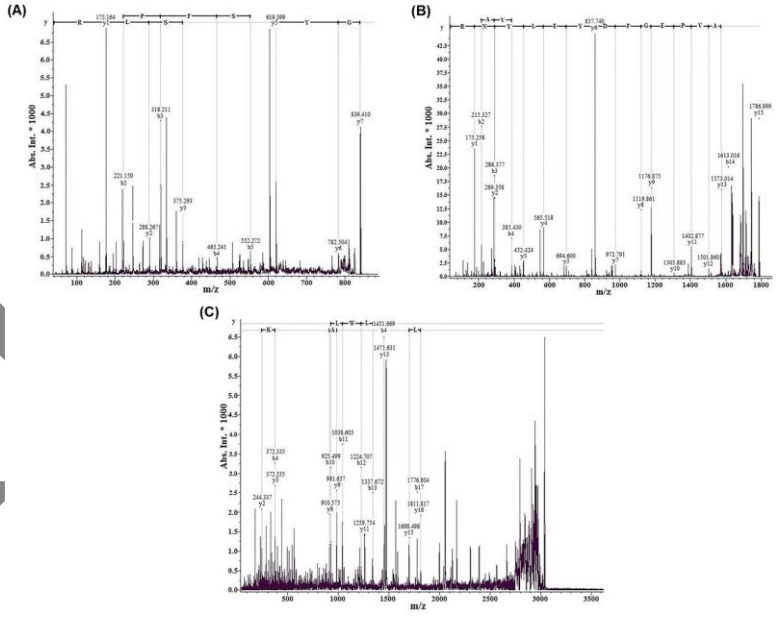

Fig. 4: MALDI-TOF-MS/MS spectra of m/z 839.410 (A), m/z 1786.999 (B) and m/z 3035.779 (C) from the PMF of the 40.0 kDa protein

Conclusion

In present study, a new protein purified from rape bee pollen had been found and was proved to be reversibly glycosylated polypeptide 1 which existed in cytoplasm, the extine of anther cell and outside of anther cell. Our results will lay a foundation for further study of the structure and biological properties of the RGP1 in the pollen development.

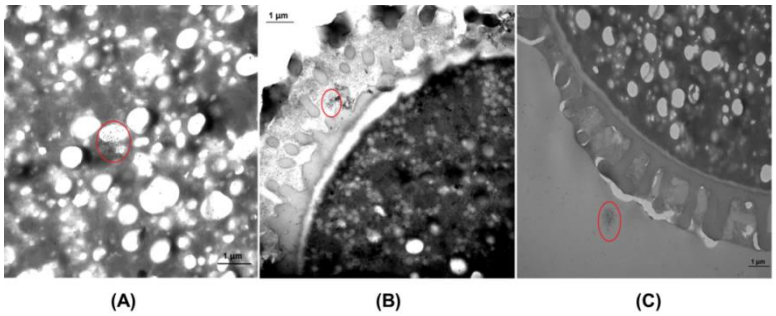

Fig. 5: TEM showing immunolocalization of RGP1 in rape anther. Immunogold particles present in cytoplasm (A), the extine (B) of the anther cell and outside of the anther cell (C)

Acknowledgments

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