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Purple acid phosphatase in the walls of tobacco cells

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ABSTRACT

Purple acid phosphatase isolated from the walls of tobacco cells appears to be a 220 kDa homotetramer composed of 60 kDa subunits, which is purple in color and which contains iron as its only metal ion. Although the phosphatase did not require dithiothreitol for activity and was not inhibited by phenylarsine oxide, the enzyme showed a higher catalytic efficiency (k_{cat}/K_m) for phosphotyrosine-containing peptides than for other substrates including *p*-nitrophenyl-phosphate and ATP. The phosphatase formed as a 120 kDa dimer in the cytoplasm and as a 220 kDa tetramer in the walls, where Brefeldin A blocked its secretion during wall regeneration. According to our double-immunofluorescence labeling results, the enzyme might be translocated through the Golgi apparatus to the walls at the interphase and to the cell plate during cytokinesis.

1. Introduction

Purple acid phosphatases (PAPs) are distinguished from other acid phosphatases by their purple color, which is due to a tyrosine to Fe(III) charge transfer transition (Antanaitis et al., 1983), and by their insensitivity to tartrate inhibition (Doi et al., 1988). The first plant PAP to be described was an enzyme from the red kidney bean, a homodimeric glycoprotein that has a molecular mass of approximately 110 kDa and contains a Fe(III)–Zn(II) metal center in each subunit (Sträter et al., 1992; Suerbaum et al., 1993; Stahl et al., 1994). The X-ray crystal structure of the enzyme showed the Fe(III)–Zn(II) metal center coordinated by three histidines, two aspartates, a tyrosine, and an asparagine (Sträter et al., 1995; Klabunde et al., 1996). The structural analysis indicated a striking similarity to both protein phosphatase 1 (Goldberg et al., 1995; Egloff et al., 1995) and protein phosphatase 2B (calcineurin) (Griffith et al., 1995; Kissinger et al., 1995) in the coordination environments of the active sites; the major difference is the replacement of the tyrosinate ligand to a coordinated water molecule in the protein phosphatases (Funhoff et al., 2001).

Despite the wealth of structural information about PAPs, there have only been a few reports to date about their function because their substrate has not yet been determined. Mammalian PAPs exhibit a broad and non-specific activity towards phosphoproteins (Ljusberg et al., 1999; Hayman et al., 1989). It has been proposed that they are involved in iron transport (Nuttleman and Roberts, 1990), the generation of reactive oxygen species (Sibille et al., 1987) and bone resorption (Ek-Rylander et al., 1994), among other processes. We have shown that wall-bound PAP was involved in wall regeneration in the protoplasts prepared from tobacco cells (Kaida et al., 2003; Sano et al., 2003). Its main function, however, is believed to be the release of inorganic phosphates as phosphorus nutrients from phosphate esters in the soil as well as in the plant (Cashikar et al., 1997; del Pozo et al., 1999; Miller et al., 2001; Bozzo et al., 2004; Zimmermann et al., 2004; Veljanovski et al., 2006; Xiao et al., 2006).

We previously reported that purified PAP from tobacco cell walls had a mass of 60 kDa with an additional smear band of 120 kDa (Kaneko et al., 1998); this measurement was made using the reducing SDS–PAGE technique, in which the SDS sample buffer contained β-mercaptoethanol. In this paper we report that tobacco wall-bound PAP forms a tetramer of 220 kDa on SDS–PAGE in the absence of a reducing agent. Furthermore, we explain the properties of PAP isolated from the walls of cultured tobacco cells, its substrate specificity, and its translocation to the apoplastic spaces.

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2. Results and discussion

2.1. Purification and characterization of purple acid phosphatase

Ionically bound cell wall PAP was extracted from a wall preparation of suspension-cultured tobacco cells by stirring with 0.7 M NaCl, and then purified according to the purification procedure we have described elsewhere (Kaneko et al., 1998), with modifications. The acid phosphatase activity was eluted from a hydroxyapatite column. First and second butyl-Toyopearl columns were then subjected to gel filtration on Superdex 200. Table 1 summarizes the purification of the wall-bound PAP including gel filtration.

The enzyme was purified 213-fold until it reached a final specific activity of 494 U mg^{-1}. The ratio of 0.7 M NaCl-soluble wall-bound proteins to the total number of proteins in the cells (including wall proteins, cytoplasmic proteins, and proteins secreted to the culture medium) was about 0.5%, and the level of purification compared to the initial concentration was approximately 4000-fold. The highly purified enzyme was purple, and we confirmed it as NtPAP12 (Kaida et al., 2003) since its N-terminal sequence (TVDMPLDSDVFRAPPGYNA) was identical to the N-terminal sequence of the deduced amino acid sequence in NtPAP12 by analysis using DNAMAN (Lynnon Biosoft, Quebec, Canada).

In SDS–PAGE, the enzyme appeared as a single polypeptide band with a molecular mass of 220 kDa under non-reducing conditions (Fig. 1). Since only one band corresponding to a molecular size of 60 kDa was detected in the presence of dithiothreitol (DTT), the native subunits of the enzyme could exist as a 220 kDa homotetramer composed of 60 kDa subunits. The phosphatase activity was not diminished in the presence of DTT. One question that remains is how the polypeptide subunits (60 kDa) form a tetramer (220 kDa), because, according to the deduced amino acid sequence of NtPAP12, only one cysteine residue that can form a disulfide bridge is present at residue 379. One possibility is that the four subunits of the phosphatase are non-covalently bound to each other to form the tetrameric structure. It is known that the acid phosphatase from yellow lupin forms a dimer of 50 and 44 kDa subunits without S–S linkage (Olczak et al., 1997).

The characteristic purple color of the purified enzyme could be attributed to a charge transfer transition from a tyrosine, potentially Tyr 201, bound to Fe(III) in the binuclear center (Antanaitis et al., 1983). Our X-ray microanalysis system, fitted with a variable-pressure scanning electron microscope, detected Fe Kα emission as well as Kα emissions of C and O, constituent elements of biological materials (Fig. 2). The enzyme might contain a mixed-valent diiron unit in its catalytic active form, as is the case in mammalian phosphatases (Antanaitis et al., 1983) and sweet potato (Waratrujiwong et al., 2006).

Table 1
Purification of purple acid phosphatase from the walls of tobacco cells

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Recovery (%)
Crude extract	100	232.1	2.32	1	100
Hydroxyapatite	18.8	146.2	7.78	3.4	63
1st Toyopearl-butyl	0.81	69.6	85.9	37.0	30
2nd Toyopearl-butyl	0.39	51.1	131	56.5	22
Superdex 200	0.08	39.5	494	213	17

The activity was determined by using *p*-nitrophenyl phosphate as a substrate.

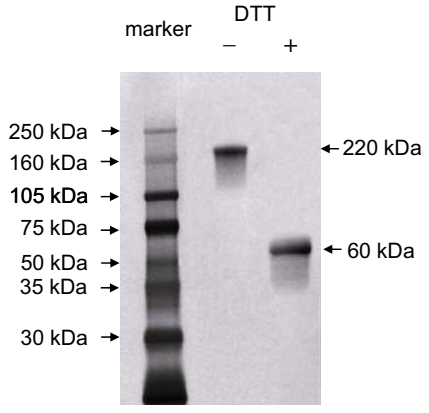

Fig. 1. SDS–PAGE of tobacco purple acid phosphatase. The purified enzyme (1 μg) was subjected to 10% SDS–PAGE in the absence (–) or presence (+) of 2 mM dithiothreitol (DTT) and stained with silver.

2.2. Enzymatic properties

As we show in Table 2, tobacco wall-bound PAP has a similar level of affinity with *p*-nitrophenyl-phosphate, ATP, phosphotyrosine, and phosphoserine, although phosphotyrosine had a higher k_{cat}/K_m value for the phosphatase than did phosphoserine, by a factor of 2.3. The phosphatase had a higher affinity and catalytic efficiency for two tyrosine phosphopeptides, ENDpYINASL (Daum et al., 1993) and DADEpYLIPQQG (Zhang et al., 1993), than for the serine/threonine phosphopeptide RRApTVA (Pinna and Ruzzene, 1996), unlike both ATP and *p*-nitrophenyl-phosphate. Gellatly et al. (1994) found a potato tuber acid phosphatase having a significant phosphotyrosine phosphatase activity. In addition, a few papers have reported findings indicating that PAPs could catalyze the hydrolysis of phosphotyrosine in tomato (Bozzo et al., 2002) and *Arabidopsis* (Veljanovski et al., 2006). Nevertheless, several phosphoproteins occur in the walls in *Arabidopsis* (Kwon et al., 2005).

Tobacco wall-bound PAP was insensitive to tartrate inhibition, qualifying it as a tartrate-resistant acid phosphatase (Doi et al., 1988) (Table 3). The activity of the phosphatase was significantly

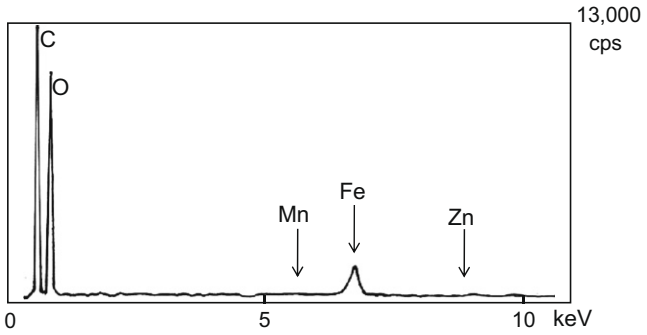

Fig. 2. X-ray spectrum of tobacco purple acid phosphatase in variable-pressure scanning electron microscopy.

Table 2
Substrate specificity of tobacco purple acid phosphatase

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
p-Nitrophenyl phosphate	0.33	125.0	379
ATP	0.40	133.2	333
IDP	1.45	60.4	42
Phosphotyrosine	0.41	62.4	152
END(pY)INASL	0.03	32.8	1093
DADE(pY)LIPQQG	0.02	26.7	1335
Phosphoserine	0.40	26.8	67
RRA(pT)VA	0.22	2.0	9

The activity was determined as the amount of phosphate released.

Table 3
Effect of various substances on the activity of purple acid phosphatase

Additive	Concentration (mM)	Relative activity[a] (%)
None		100
Vanadate	0.01	63
Vanadate	0.1	24
Vanadate	1	3
Molybdate	0.01	21
Molybdate	0.1	7
NaF	2	73
Tartrate	2	99
$ZnCl_2$	2	47
Okadaic acid	0.01	98
Microcystin LR	0.01	98
Phenylarsine oxide	2	93
$CaCl_2$	5	94
$MgCl_2$	5	98
$FeCl_2$	5	97
$FeCl_3$	5	85
$MnCl_2$	5	98
EDTA	2	103
Dithiothreitol	2	93

[a] The activities represent the mean of three assays carried out for each sample.

inhibited by Zn^{2+}, molybdate, and orthovanadate, but it was not inhibited by phenylarsine oxide. Orthovanadate inhibits all protein tyrosine phosphatases at concentrations between 10 and 100 μM, while molybdate, phenylarsine oxide, and Zn^{2+} differentially inhibit protein tyrosine phosphatases (Walton and Dixon, 1993). Okadaic acid and microcystin-LR, which inhibit protein phosphatases 1, 2A, and 2B (Takai et al., 1987; MacKintosh et al., 1990), had no effect on the activity of wall-bound PAP; neither did Ca^{2+} or Mg^{2+}, which are required for the activity of protein phosphatase 2B and protein phosphatase 2 C, respectively (Cohen, 1989). The activity of wall-bound PAP, which is sensitive to vanadate, insensitive to okadaic acid, and does not require Ca^{2+} and Mg^{2+}, seems to be similar to that of protein tyrosine phosphatases (Luan, 2003).

Purple acid phosphatase is not classified among the protein phosphatases (protein tyrosine phosphatase and protein serine/threonine phosphatase), although the structures of the coordination environments of the active sites of PAPs are similar to those of serine/threonine phosphatases. In our study, however, wall-bound PAP showed a higher catalytic efficiency (k_{cat}/K_m) for phosphotyrosine-containing peptides than for other substrates including *p*-nitrophenyl-phosphate and ATP. Therefore, it is possible that phosphotyrosine protein could be the substrate for wall-bound PAP.

2.3. Translocation of PAP into the walls

Western blot analysis showed that the phosphatase occurred as a 120 kDa polypeptide in the cytoplasm and as a 220 kDa polypeptide in the walls during wall regeneration (Fig. 3). This showed that the tetramer could be formed from phosphatase subunits in the walls. During the regeneration of cell walls in tobacco protoplasts, PAP increased in the walls during cultivation while the level of protein in the cytoplasm remained constant. Upon the addition of Brefeldin A (10 μg/ml), PAP in the walls was found to have decreased after 30 min of cultivation. This observation showed that Brefeldin A might block the secretion of PAP in protoplasts during wall regeneration under conditions of high turnover for the phosphatase. This is in agreement with our previous observation showing the inhibitory effect of Brefeldin A on the secretion of acid phosphatase activity in protoplasts regenerating their cell walls, in which Brefeldin A caused the formation of stacks of hybrid ER-Golgi membranes in tobacco protoplasts (Kaneko et al., 1996).

A double-immunofluorescence labeling experiment was performed using rabbit anti-wall-bound purple acid phosphatase antibody and JIM 84 for Golgi (Knox et al., 1989; Horsley et al., 1993). The labeling pattern of the phosphatase was identical to that of JIM 84, showing the localization of PAP in the Golgi apparatus at the interphase of cells during the cell cycle (Fig. 4A). PAP was also observed to localize in the Golgi at their metaphase before cell plate formation (Fig. 4B). These results indicate that purple acid phosphatase might be translocated through the Golgi apparatus to the walls at the interphase and to the cell plate during cytokinesis. We have concluded that wall-bound PAP occurs as a 120 kDa dimer polypeptide in the Golgi, and that this dimer forms a 220 kDa tetramer in the walls during wall translocation.

3. Experimental methods

3.1. Culture of tobacco cells

The tobacco cell line XD-6 derived from *Nicotiana tabacum* L. var. Xanthi was cultured in Murashige and Skoog medium (40 ml) containing 3% sucrose and 2,4-dichlorophenoxyacetic acid (1 mg L^{-1}) in a 100-ml flask at 25 °C in the dark.

Five or 6-day-old cells in the logarithmic phase of growth were used for all experiments.

3.2. Purification of wall-bound PAP from tobacco cells

Wall-bound PAP was purified according to the purification procedure described by Kaneko et al. (1998), with modifications. Suspension-cultured tobacco cells (500 g) were homogenized in 50 mM HEPES-KOH (pH 7.0) buffer using a Teflon-glass homogenizer at 1500 rpm for 20 min. The insoluble wall materials were collected on nylon-mesh (pore size, 50 μm) and washed, not only 5 times with 10 mM HEPES–KOH buffer (pH 7.0) by centrifugation at 5000 × *g* for 20 min, but also twice with 10 mM HEPES–KOH (pH

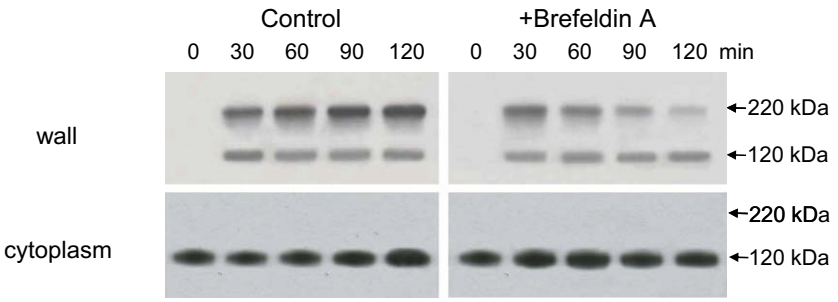

Fig. 3. Immunoblot analyses of the wall and cytoplasmic fractions extracted from protoplasts regenerating walls. The protoplasts were cultured in the medium for wall regeneration in the absence (control) or presence (+Brefeldin A) of Brefeldin A and harvested at the indicated times after the onset of culture. Five micrograms of total protein were applied to SDS–PAGE under non-reducing conditions followed by immunoblot analyses.

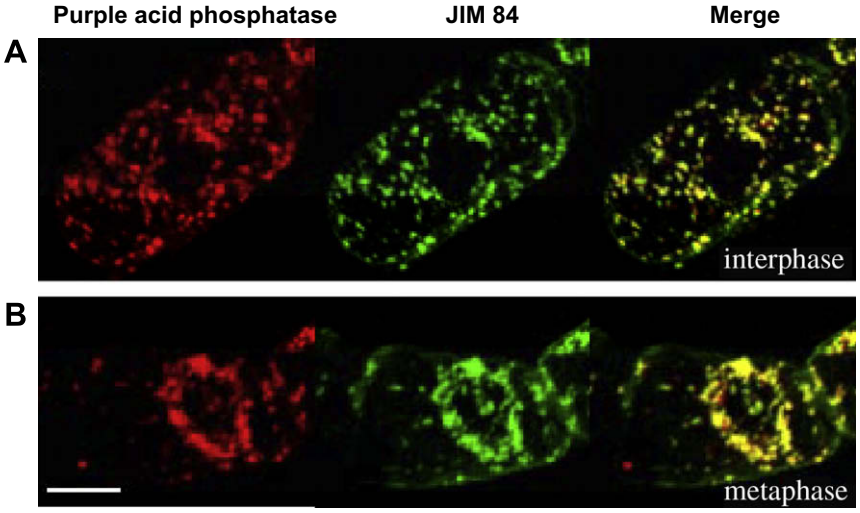

Fig. 4. Confocal immunofluorescence micrographs of purple acid phosphatase and Golgi apparatus in the interphase (A) and metaphase (B) of tobacco cells during the cell cycle. Purple acid phosphatase and Golgi apparatus were stained with anti-wall-bound purple acid phosphatase antibody and JIM 84, respectively. Bar = 10 μm.

7.0) containing 0.2 M NaCl. The wall-bound phosphatase was extracted with the same buffer containing 0.7 M NaCl. The extract was concentrated by precipitation with $(NH_4)_2SO_4$ at 65% saturation. Following centrifugation at 14,000 × *g* for 30 min, the pellet was dissolved in 10 mM Tris–HCl (pH 7.0) and dialyzed against the same buffer. The sample was loaded onto a column (20 × 250 mm) of hydroxyapatite (Seikagaku, Tokyo, Japan) pre-equilibrated with 250 mM Tris–HCl (pH 7.0). The column was washed with 200 ml of 250 mM Tris–HCl (pH 7.0), and then acid phosphatase activity was eluted at a flow rate of 0.2 ml/min with 150 ml of 3 M Tris–HCl (pH 7.0). The fractions (30 ml) containing activity were pooled, concentrated with $(NH_4)_2SO_4$ at 65% saturation, and dialyzed against 10 mM Tris–HCl (pH 7.0) containing 30% saturated $(NH_4)_2SO_4$. The sample was applied on a column (20 × 220 mm) of Toyopearl-butyl (Tosoh, Tokyo, Japan) pre-equilibrated with 10 mM Tris–HCl (pH 7.0) saturated 30% $(NH_4)_2SO_4$. Stepwise elution was performed with the buffer by decreasing the concentration of ammonium sulfate (200 ml of 30%, 100 ml of 20%, and 100 ml of 0%). Acid phosphatase activity was eluted at a flow rate of 0.2 ml/min with 20% $(NH_4)_2SO_4$. The fractions (15 ml) containing activity were pooled, and precipitated with 65% $(NH_4)_2SO_4$. This step was carried out twice, once with the first Toyopearl-butyl and once with the second Toyopearl-butyl. The sample was dissolved in 1 ml of 10 mM Tris–HCl (pH 7.0) containing 1 M NaCl, and applied onto a Superdex 200 HR 10/30 column (10 × 300 mm) (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) pre-equilibrated with 10 mM Tris–HCl (pH 7.0) containing 1 M NaCl. The activity was eluted at a flow rate of 2.0 ml/min with 100 ml of 10 mM Tris–HCl (pH 7.0) containing 1 M NaCl. The fractions containing phosphatase activity were precipitated with 65% $(NH_4)_2SO_4$ and the precipitate was stored at −80 °C.

3.3. Enzyme assays

For the purification procedure, acid phosphatase activity was determined by the hydrolysis of *p*-nitrophenyl phosphate. The reaction mixture contained an enzyme preparation, 1 mM *p*-nitrophenyl phosphate and 50 mM sodium acetate buffer (pH 5.8) in a total volume of 0.1 ml. After incubation at 30 °C for 3 min, the reaction was stopped by addition of Na_2CO_3. One unit was defined as 1 μmol of *p*-nitrophenyl phosphate hydrolyzed per min. For the determination of kinetic parameters, acid phosphatase activity was measured as the amount of phosphate released from substrate, according to the method described by Van Veldhoven and Mannaerts (1987). Each assay contained substrate and 50 mM sodium acetate buffer (pH 5.8) in a total volume of 0.1 ml. Reactions were stopped at 3 min by the addition of Molybdate Dye/Additive mixture (Promega, Madison, WI, USA). Controls were carried out using the background levels of phosphate present at each substrate concentration. One unit of activity is defined as the amount of enzyme necessary to produce 1 μmol of phosphate per 1 min at 30 °C. Protein content was quantified according to the method described by Bradford (1976) using bovine serum albumin as the standard.

3.4. Electrophoresis

SDS–PAGE was performed with 10% acrylamide minigel according to the manufacturer's instructions (Atto, Tokyo Japan). For the determination of molecular mass, a plot of log molecular mass versus retention factor (R_f) value was constructed using the standard proteins of molecular weight marker (GE Healthcare, USA). Reducing and non-reducing conditions corresponded to the sample treatment with SDS at 80 °C for 15 min in the presence and absence of DTT.

3.5. Sequencing of the polypeptide

After SDS–PAGE was completed, the proteins were electrotransferred to PVDF membrane. The membrane was stained with Coomassie Blue and the stained polypeptide was subjected to sequencing from the N-terminus with a protein sequencer (Applied Biosystems, 477A, Foster City, CA, USA).

3.6. Determination of metal ion

The metal ion of tobacco wall-bound PAP was analyzed using an EDX system (Horiba, EMAX-7000, Kyoto, Japan) fitted with a variable-pressure scanning electron microscope (VP-SEM) (Hitachi, S-3500N, Tokyo, Japan). The chamber pressure was 30 Pa. The accelerating voltage was 15 kV.

3.7. Preparation and culture of protoplasts

The preparation and culture of the protoplasts were performed according to the procedure described in a previous report (Kaida et al., 2003). Cells were incubated for 40 min at 30 °C with a

mixture of 1.5% (W/V) cellulase (Cellulase Onozuka RS, Yakult, Tokyo, Japan), 0.05% (W/V) pectinase (pectolyase Y-23, Seishin, Tokyo, Japan), and 0.48 M mannitol at pH 5.2 to digest the cell walls. The protoplasts were washed by centrifugation at 800 × *g* for 2 min in 0.48 M mannitol, and then incubated in the medium described by Nagata and Takebe (1970) at a density of 10^5 protoplasts per ml at 26 °C. BFA (Wako, Osaka, Japan) was added to a final concentration of 10 μ/ml in the medium at the start of culture.

3.8. Immunoblotting analysis

The protoplasts (2 ml) were washed by centrifugation at 800 × *g* for 2 min in 0.48 M mannitol, lysed in 10 mM HEPES–KOH buffer (pH 7.0) and then centrifuged at 3000 × *g* for 10 min to separate the cytoplasmic and wall fractions. Proteins were separated by SDS–PAGE, electrotransferred to Hybond-ECL (GE Healthcare), and probed first with rabbit anti-wall-bound PAP antibody and then with peroxidase-labeled second antibody. Detection was performed using ECL Western Blotting Detection Reagents (GE Healthcare). The polyclonal antibody against PAP was obtained by immunizing two rabbits. One hundred and fifty micrograms of the enzyme was injected into each rabbit per one immunization. A complete adjuvant was used for the first injection and incomplete adjuvant was used after second injections. We performed the immunization six times every 2 weeks and took a blood sample at 7 days after the final immunization.

3.9. Immunofluorescence staining

The localization of the Golgi apparatus and intracellular PAP were observed using the method described by Yoneda and Hasezawa (2003), with modifications. The BY-2 cells were placed onto coverslips coated with poly-L-lysine (M.W. 70,000–150,000, SIGMA, Croydon, Australia). After 5 min, the cells were treated with 0.25% (w/v) cellulase (Cellulase Onozuka RS, Yakult) and 0.05% (w/v) pectinase (pectolyase Y-23, Seishin) in PMEG solution (50 mM PIPES, pH 6.8, 1 mM $MgSO_4$, 5 mM EGTA, and 1% glycerol) for 5 min, followed by washing with the same buffer. The cells were fixed with 3.7% (w/v) formaldehyde in the PMEG solution for 1 h and treated with 0.5% (v/v) Triton X-100 in the PMEG solution for 20 min to give them permeability; then they were washed with phosphate-buffered saline (PBS, 20 mM Na-phosphate, pH 7.0 and 150 mM NaCl). Prior to immunostaining, the cells were treated with 1% (w/v) BSA, 0.1 M glycine, and 0.05% Triton X-100 in PBS for 20 min.

For double-immunofluorescence labeling, the cells were first incubated with JIM 84 monoclonal antibody for 4 h, washed with PBS, and then incubated with rabbit anti-wall-bound PAP antibody for 1 h. After a second washing with PBS, the cells were incubated with Alexa Fluor 488 conjugated anti-mouse IgM and Alexa Fluor 568 conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) for 1 h, then washed a third time with PBS. The cells were subsequently embedded in an antifading reagent (SlowFade Light; Invitrogen) and observed under a fluorescence microscope (Olympus BX50, Tokyo, Japan) equipped with a confocal laser scanning head system (Leica TCSNT, Solms, Germany).

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