Characterization of Phosphatidylinositol-Specific Phospholipase C (PI-PLC) from Lilium daviddii Pollen

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The phosphatidylinositol-specific phospholipase C (PI-PLC) activity is detected in purified Lilium pollen protoplasts. Two PI-PLC full length cDNAs, LdPLC1 and LdPLC2, were isolated from pollen of Lilium daviddii. The amino acid sequences for the two PI-PLCs deduced from the two cDNA sequences contain X, Y catalytic motifs and C2 domains. Blast analysis shows that LdPLCs have 60–65% identities to the PI-PLCs from other plant species. Both recombinant PI-PLCs proteins expressed in E. coli cells show the PIP2-hydrolyzing activity. The RT-PCR analysis shows that both of them are expressed in pollen grains, whereas expression level of LdPLC2 is induced in germinating pollen. The exogenous purified calmodulin (CaM) is able to stimulate the activity of the PI-PLC when it is added into the pollen protoplast medium, while anti-CaM antibody suppresses the stimulation effect caused by exogenous CaM. PI-PLC activity is enhanced by G protein agonist cholera toxin and decreased by G protein antagonist pertussis toxin. Increasing in PI-PLC activity caused by exogenous purified CaM is also inhibited by pertussis toxin. A PI-PLC inhibitor, U-73122, inhibited the stimulation of PI-PLC activity caused by cholina toxin and it also leads to the decrease of [Ca2+]c in pollen grains. Those results suggest that the PPI-PLC signaling pathway is present in Lilium daviddii pollen, and PI-PLC activity might be regulated by a heterotrimeric G protein and extracellular CaM.

Keywords: cDNA cloning — Extracellular calmodulin (CaM) — Heterotrimeric G protein — Lilium daviddii pollen — Phosphatidylinositol-specific phospholipase C.

Abbreviations: CaM, calmodulin; CTX, cholera toxin; DAG, diacylglycerol; IP3, inositol-1,4,5-trisphosphate; IP3R, IP3 receptor; PI-PLC, phosphoinositide-specific phospholipase C; PIP2, phosphatidylinositol-4,5-bisphosphate; PTX, pertussis toxin; U73122, (1-[6-(amino)hexyl]-2,5-pyrrolidinedione); U73343, (1-[6-(amino)hexyl]-2,5-pyrrolidinedione). The nucleotide sequences reported in this paper have been submitted to the GenBank database under the following accession numbers: AY735314 (LdPLC1) and AY735313 (LdPLC2).

Introduction

The phosphatidylinositol-specific phospholipase C (PI-PLC) signaling pathway has been implicated in a variety of physiological processes in both animals and plants. Many extracellular signals are transmitted across the plasma membrane by PI-PLC, which hydrolyzes the phosphoinositide 4,5-bisphosphate (PIP2), a minor membrane phospholipids, and result in the generation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), both as novel second messengers in plant cells (Meijer and Munnik 2003). In the plant system, PI-PLC activity was first identified in oat root plasma membranes (Tate et al. 1989). From then on, the characterization of PI-PLC has been widely studied (Melin et al. 1992, Cho et al. 1995, Huang et al. 1995, Shi et al. 1995a, Brearley et al. 1997). Yamamoto et al. (1995) cloned the first plant EST from Arabidopsis thaliana shoots during floral induction, which matched to the PI-PLC. Hirayama et al. (1995) obtained a cDNA (AtPLC1) from Arabidopsis thaliana shoots under dehydration and salt stress conditions; while AtPLC2 is constitutively expressed in vegetative and floral tissues in Arabidopsis thaliana (Hirayama et al. 1997). The cDNAs of PI-PLC have been cloned in many kinds of plant so far, such as soybean (Shi et al. 1995b), potato (Kopka et al. 1998), pea (Venkataraman et al. 2003), mung bean (Vigna radiata L.) (Kim et al. 2004) and moss Physcomitrella patens (Mikami et al. 2004). Furthermore, a series of studies suggest that plant PI-PLCs, as in animals, play important roles in a wide variety of physiological processes. They are involved in response to a variety of stimuli, including environmental stress (Legendre et al. 1993, Smolenska-Sym and Kacperska 1996, Chapman 1998), gravity (Perera et al. 1999), anaerobic stress (Reggiani and Laoret 2000), pathogen attack and pollination (Stevenson et al. 2000, Wang 2001) and aluminium (Al) stress (Martinez-Estevez et al. 2003), especially in ABA signal pathway (Assmann and Shimazaki 1999, Staxen et al. 1999, Sanchez and Chua 2001, Cousson et al. 1999, Henriques et al. 2001, Hunt et al. 2003, Mills et al. 2004). Recently, plant PI-PLCs were also verified to be involved in cytokinin and gravity responses (Repp et al. 2004) and the response to ELF-EMF (Piacentini et al. 2004).

Pollin germination and tube growth are complicated and hazardous in the fertilization process and many factors involved in this process remain to be identified (Franklin-Tong...
Phospholipase C pathway in pollen

Fig. 1 Detection of PI-PLC activity in Lilium daviddi pollen protoplasts. Protoplasts were incubated with authenticated [3H]-PIP2, and the release of IP3 was measured. The data are from three independent experiments; vertical bars indicate standard error range.

Results

Analysis of PI-PLC activity in the Lilium daviddi pollen protoplasts

To measure the PI-PLC activity directly, the pollen protoplasts were prepared according to the method of Tanaka et al. (1987), and those protoplasts were used to measure the PI-PLC activity in the pollen. The PIP2-dependent PI-PLC activity was detected in pollen using the method of Melin et al. (1992). It was found that the PIP2-dependent PI-PLC activity increased linearity for the first hour and then became stable (Fig. 1).

Cloning of PI-PLC cDNA from Lilium daviddi pollen

The above mentioned results suggest the presence of PI-PLC transcript in Lilium daviddi pollen. In order to clone the PLC gene in Lilium daviddi pollen, two degenerate oligonucleotides designed according to the X domain conserved amino acid sequence were used in a PCR analysis with cDNA from Lilium daviddi pollen as a template. Two DNA fragments with about 400 bp in length were amplified. Blast analysis indicated that these two fragments were homologous to plant PI-PLCs. The nested primers were designed according to the two fragment nucleotide sequences and the two full length cDNAs corresponding to the two PI-PLCs were obtained by 5′RACE and 3′RACE, and they were named as LdPLC1 and 2. The full-length cDNA of LdPLC1 has 1878bp with an open reading frame representing 475 amino acids, and 1785bp cDNA of LdPLC2 codes a protein with 536 amino acids in length. Relative molecular weights of Lilium daviddi PI-PLC enzymes are 54 kDa and 61 kDa, respectively. The pls are 8.572 (LdPLC1) and 5.978 (LdPLC2), respectively. BLAST results indicate that the primary structures of LdPLC1 and LdPLC2 are similar to those of currently known plant PI-PLC homologs (http://www.ebi.ac.uk/clustalw). Homology analysis among some plants’ PI-PLC protein sequences indicate that LdPLC1 and LdPLC2 are more closely related to each other than to any other plants’ PI-PLCs (Fig. 2 and supplemental Fig. 1) and they show 60–65% identities to other plants’ PI-PLCs (supplemental Fig. 1). The deduced amino acid sequences of LdPLCs include the X, Y and C2-domains, which are ubiquitous in other PI-PLCs, but N termini of LdPLCs are shorter than the primary structures of other plant species.

The recombinant protein of LdPLC possesses PIP2-hydrolyzing activity

To confirm that the LdPLCs genes cloned from Lilium daviddi pollen encode the proteins with PI-PLC activity, we introduced the full length cDNA of LdPLCs into pET28b vector. The recombinant protein of AtPLC1 expressed in E. coli was used as a positive control, which had showed the PI-PLC activity as described by Hirayama et al. (1995). Both...
recombinant LdPLCs and AtPLC1 expressed in E. coli BL21 cells contained a 6-His-tag in the N-terminal, and they showed the expected electrophoretic motilities for the relative molecular weights predicted from the cDNA sequences (data not shown).

Four kinds of crude extracts of E. coli cells (induced by IPTG) including harbored pET28b vector (negative control), pET28b-AtPI-PLC1 (positive control), pET28b-LdPLC1 and pET28b-LdPLC2, were detected for PIP2-hydrolyzing activity. It was shown that both LdPLCs possessed PIP2-hydrolyzing activity, while no PI-PLC activity was detected in the negative control (Fig. 3). Compared with the positive control AtPLC1, the PIP2-hydrolyzing activity for LdPLC1 was lower, but LdPLC2 was higher than that of AtPLC1 (Fig. 3). Those results suggest that both of the two LdPLCs we cloned from Lilium daviddii pollen have PI-PLC activity.

Analysis of PI-PLC genes expression in Lilium daviddii pollen grains and pollen protoplasts

To assess whether the two LdPLCs are related to pollen germination, we first check the expression pattern of LdPLCs in the pollen by RT-PCR. The results show that both LdPLC1 and LdPLC2 are expressed in both pre-germinated and germinating pollen. The transcript level did not change during pollen germinating process (Fig. 4A). LdPLCs were also expressed in the leaf (data not shown), suggesting that they are not specifically expressed in pollen.

In order to assess whether the protoplasting process affect LdPLCs expression, the transcription levels of LdPLCs from pollen grains and pollen protoplasts are compared. It was shown that there was not obvious different for LdPLCs expression level between pollen grains and pollen protoplasts, suggesting that protoplasting process may not affect LdPLCs expression (Fig. 4B).

Regulating factors of PI-PLC activity in the Lilium daviddii pollen protoplasts

Our previous work indicated that extracellular CaM was able to regulate pollen germination and tube growth (Ma and Sun 1997), a heterotrimeric G protein might be involved in the regulation of pollen tube growth and extracellular CaM was able to activate intrinsic GTPase activity of the heterotrimeric G protein in pollen (Ma et al. 1999). To assess the relationship among extracellular CaM, G protein and PI-PLC in the regulation of pollen germination and tube growth, we measured the effect of exogenous CaM and heterotrimeric G protein on the PI-PLC activity by supplying with exogenous purified CaM or G protein modulators into the incubated pollen protoplast system. It was found that the PI-PLC activity was increased by adding exogenous CaM in a dose-dependent manner, whereas a control protein, BSA, had no effect on the activity of PI-PLC (Fig. 5A). The stimulation effect increased about three-fold when the exogenous CaM concentrations were higher than 10^{-9} mol liter^{-1} (Fig. 5A). Anti-CaM antibody inhibited the stimulation effect of PI-PLC activity caused by exogenous CaM in a dose-dependent manner (Fig. 5B).

Using G protein modulators, we addressed the relationship between PI-PLC and heterotrimeric G protein activities, and we observed that the activity of PI-PLC was increased by G protein agonist cholera toxin (CTX) and inhibited by G protein antagonist pertussis toxin (PTX) (Fig. 6). PI-PLC activity was stimulated by CTX at the range from 200 to 800 ng ml^{-1}, and it was enhanced about 3-fold when the CTX concentration was at 600 ng ml^{-1} (Fig. 6A). Whereas, PI-PLC activity was inhibited by PTX at a concentration from 400 to 800 ng ml^{-1} in a dose-dependent manner (Fig. 6B). Those results suggest that a hetero-
otrimeric G protein might be an upstream-regulator of PI-PLC activity.

These results led us to predict that extracellular CaM, heterotrimeric G protein and PI-PLC might work in the same signal transduction pathway. Therefore, the relationship between extracellular CaM and the heterotrimeric G protein in the regulation of PI-PLC activity in pollen were further investigated. We found that the stimulation effect of extracellular CaM on PI-PLC activity was inhibited by PTX in a dose-dependent manner (Fig. 7A), suggesting that heterotrimeric G protein acted downstream of extracellular CaM for the regulation of PI-PLC activity. It was also found that the stimulation effect of CTX on PI-PLC activity was inhibited by PI-PLC antagonist U-73122, whereas a weaker PI-PLC antagonist U-73343 showed a less inhibitory effect compared with that of U-73122 (Fig. 7B). This result may further indicate that PI-PLC acts downstream of heterotrimeric G protein.

The effects of membrane-permeable PI-PLC antagonist on the [Ca\(^{2+}\)]\(_{cyt}\) in pollens

To verify whether PI-PLC activity is necessary for [Ca\(^{2+}\)]\(_{cyt}\) maintaining, we checked the [Ca\(^{2+}\)]\(_{cyt}\) using confocal laser scanning microscope. We observed an obvious polar distribution (data not show) and weak oscillation of [Ca\(^{2+}\)]\(_{cyt}\) in intact Lilium davidii pollen grains (Fig. 8A, B, control curve). Shortly after U-73122 was applied to the bathing medium, [Ca\(^{2+}\)]\(_{cyt}\) decreased slowly during the following 30 min, no apparent [Ca\(^{2+}\)]\(_{cyt}\) changes were able to be recorded in the control and in U-73343 treatment (Fig. 8A).

Our previous evidence suggested that the purified exogenous CaM could elevate the intracellular calcium concentration (Shang et al. 2001). In this study, we observed that the effect of exogenous CaM on [Ca\(^{2+}\)]\(_{cyt}\) increase was inhibited by U-73122 when both of them were added in the bathing medium (Fig. 8B). These results further suggest that PI-PLC may be involved in the regulation of [Ca\(^{2+}\)]\(_{cyt}\) in pollen.
The presence of PI-PLC gene and PI-PLC enzyme activity in the Lilium daviddi pollen

In mammalian cells, eleven distinct PI-PLCs, which are grouped into four subfamilies as PI-PLCβ, γ, δ and ε, have been identified. There are five domains which are conserved in animal PI-PLC enzymes, these domains include: PH domain in the N-terminus, an EF-hand domain, an X domain, a Y domain which together with X domain to constitute the catalytic domain of the enzyme, and a C2 domain in the C-terminus (Wang 2001). Recently, a novel, sperm-specific phospholipase C, PLCζ, was identified from mouse (Saunders et al. 2002), it triggers Ca^{2+} oscillations at fertilization and plays a role as the molecular trigger for development of a fertilized egg into an embryo. PLCζ lacks PH domain compared with the typical animal PI-PLCδ type. Plants’ PI-PLCs appear to have only one gene family, which is similar to the PI-PLCδ subfamily of mammalian PI-PLC family (Wang 2001). In this paper, we isolated two full length cDNA sequences of PI-PLC from Lilium daviddi pollen. BLAST analysis show that both LdPLCs had three domains, X, Y and C2 domain, which are core sequences found in typical PI-PLCs, and they show 60–65% sequence identities to other plants’ PI-PLCs (Fig. 2). Different from other plants PI-PLC, LdPLC2 has shorter EF-hand domain in N-terminus, and LdPLC1 absents EF-hand domain. The similar molecular structure appears in Vr-PLC3 from mung bean, which also contains a short EF-hand (Kim et al. 2004).
Our previous works also provided some preliminary evidence for the presence and function of a PI-PLC pathway in pollen germination and tube growth (Ma et al. 1998, Wang et al. 2000). Herein we provide two more experimental evidences to show that there is a correlation between the PI-PLC activity and the germination of pollen.

First, two full-length of PI-PLC cDNAs (LdPLC1 and LdPLC2) were cloned from Lilium daviddii pollen, and RT-PCR analysis verified that both of them expressed in pollen and the expression of LdPLC2 was induced during pollen germination or tube growth process (Fig. 4). Second, the PI-PLC activities were detected in pollen protoplasts (Fig. 1). Even though we can not provide direct evidence that the PI-PLC activity we measured in the pollen came from the two LdPLCs we cloned from Lilium daviddii pollen due to the lack of Ld-PLC mutants in Lilium daviddii, the recombinant proteins of the LdPLCs expressed in E. coli cells showed the PIP2-hydrolyzing activity (Fig. 3). These results still indicate the presence and involvement of PI-PLC to regulate pollen germination and tube growth.

**Pollen PI-PLC activity is regulated by heterotrimeric G protein and exogenous CaM**

In mammalian cells, PI-PLCβ is activated by heterotrimeric G protein and PI-PLCγ is activated by tyrosine kinase-coupled receptors. Both of them play key roles in transducing extracellular signals for regulation of growth, cell proliferation, metabolism and secretion (Wang 2001). Although the sequences of LdPLCs we found, as does the other plants’ PI-PLC cDNAs, are more similar to the PI-PLCβ subfamily than other subfamilies in animals structurally (Hartweck et al. 1997, Hirayama et al. 1995, Hirayama et al. 1997, Kopka et al. 1998), there are still many evidences to support that the heterotrimeric G protein, which activates the PI-PLCβ subfamily in animals, might be involved in the regulation of plants’ PI-PLCs (Arz and Grambow 1994, Munnik et al. 1998, van Himbergen et al. 1999, Reggiani and Laoreti 2000, Millner 2001, Apone et al. 2003).

Our previous work indicated that a heterotrimeric G protein might be involved in the regulation of pollen tube growth and exogenous CaM was able to activate intrinsic GTPase activity of heterotrimeric G protein in pollen (Ma et al. 1999). In the present study, we further assess whether or not the PI-PLC activity is influenced by G protein and extracellular CaM. Due to the inefficient permeation of PIP2 into Lilium daviddii pollen grains cell through the pollen cell wall, we have to prepare the pollen protoplasts system to detect the PI-PLC activity. These results show that PI-PLC activity is increased by the treatment of G protein agonist CTX, and the increase in PI-PLC activity caused by CTX is inhibited by PI-PLC inhibitor (Fig. 6A, 7B). Whereas, PI-PLC activity is inhibited by G protein antagonist PTX (Fig. 6B). Thus, these results suggested that Lilium daviddii pollen PI-PLC activity may be regulated by heterotrimeric G protein and PI-PLC is downstream of heterotrimeric G protein in the signal transduction pathway. We also found that exogenous CaM was able to activate the PI-PLC activity (Fig. 5). These results lead us to predict that exogenous CaM, heterotrimeric G protein and PI-PLC might work in the same signal transduction pathway. Because of the stimulation effect of extracellular CaM on PI-PLC activity was inhibited by PTX in dose-dependent manner (Fig. 7A), it is suggesting that heterotrimeric G protein might act downstream of extracellular CaM for regulation of PI-PLC activity.

It should be mentioned that all PI-PLC activity data in this study are from pollen protoplasts, but neither from pollen grains nor from pollen tube. While, there is no evidence to show that pollen protoplasts are physiologically identical to pollen grains or pollen tube. The protoplasting process may activate PI-PLC proteins or induce expression of additional PI-PLC genes, which contribute toward the PI-PLC activities. Thus, the results from protoplasts may not be necessarily relevant to pollen germination.

**The PI-PLC pathway regulates mobilization of [Ca2+]/cyt in pollen**

Increase in cytosolic free Ca2+ concentration ([Ca2+]cyt) is necessary for pollen germination (Song et al. 1998), and PI-PLC pathway is involved in the release of free Ca2+ into cytosol from organelles (Rhee 2001, Wang 2001). Several evidences support that PI-PLC-IP3 signaling system is involved in mobilization and oscillation of [Ca2+]cyt in plants (Kashem et al. 2000, Wang 2001). In the pollen system, Franklin-Tong et al. (1996) showed that IP3 could induce the release of Ca2+ to regulate Papaver rhoas pollen tubes growth. Our previous work provided evidence that exogenous CaM treatment was able to increase [Ca2+]cyt in the pollen system (Shang et al. 2001). In the present study, we showed that the increase effect on [Ca2+]cyt caused by exogenous CaM was repressed by PI-PLC inhibitor (Fig. 8). These results suggest that the Ca2+ release may be the next step of the PI-PLC pathway in the pollen system. Our previous results also showed that pollen tube growth was inhibited by microinjection of IP3R antibody, and increased by microinjection of IP3 (Wang et al. 2000). Based on all the above mentioned results, we can presume that the PI-PLC-IP3-IP3R-Ca2+ cascade are involved in regulating the growth of pollen tube.

**Materials and Methods**

**Plant materials**

Pollen of Lilium daviddii was used in this study. The pollen grains were collected from freshly opened anthers and stored at −74°C for use in each experiment.

**Preparation of calmodulin and anti-calmodulin antibody**

Cauliflower calmodulin (CaM) was purified by Phenyl-Sepharose 4B affinity chromatography as described previously (Biro et al. 1984). Anti-CaM antibody was raised against potato CaM isoform V1 (PCM6), which was expressed and purified from engineering E. coli BL21 (a kindly gift from Professor Poovaiyah, Washington Univer-
Isolation and purification of protoplasts

Protoplasts were prepared by the method described by Tanaka et al. (1987), with some modifications. Pollen grains were directly suspended in White’s medium supplemented with sucrose (0.6 M), PVP (0.1%), cellulase R-10 (1.5% w/v), and 0.5% potassium dextran sulfate (all reagents are from Calbiochem), with the pH adjusted to 5.8. The enzyme treatment was performed at 30°C for 3 h on a revolving platform shaker operating at 80 rpm. After the enzyme treatment, protoplasts were washed 3 times with modified White’s solution containing 0.6 M sucrose by centrifugation at 500g for 5 min. The final suspension was then layered on a 16% Percoll solution containing the culture medium for purification. The purity of protoplasts was about 90%.

Detection of PI-PLC activity

Assay of PI-PLC activity was conducted as described by Melin et al. (1992) with some modifications. The standard incubation mixture contained 50 mM Tris–HCl, pH6.0, 0.6 M sucrose, 0.8 mM EDTA, 0.8 mM CaCl2 (25 μM free Ca2+), 10 μl [3H]-PIP2 (1,100 dpm nmol-1), NEN® in micellar solution and an appropriate amount of purified protoplasts (about 1 μg membrane protein) or the crude extract of E. coli cells (about 10 μg proteins) in a final volume of 60 μl. The [3H]-PIP2 solution was prepared by evaporating the lipids in solvent to dryness under a stream of nitrogen followed by sonication in a water bath for 10 min in Tris–HCl (50 mM pH 6.0) buffer. The above mixture was incubated at 4°C for 4 h, then a reaction was started by the addition of various agonists (ddH2O as a control) performed at 30°C for 20 min. The reaction was stopped by the addition of 750 μl chloroform/methanol (2:1, v/v) followed by 250 μl of 0.6 M HCl. After vortexing and 3 min centrifugation (2,000*g), 350 μl of the upper phase was transferred to a scintillation vial, supplemented with 5 ml of liquid (0.3 mg ml-1 POPOP, 4 mg ml-1 PPO, tolune : ethanol 7:3, v/v) and radioactivity was measured in a liquid scintillation counter (PACKARD, 2200CA). Values presented have been recalculated to correspond to the total upper phase. The analyses were performed in duplicate when the reaction rate was proportional to incubation time and amount of protein.

Preparation of RNA samples

One hundred micrograms of Lilium daviddi pollen grains were ground in a mortar with a pestle in the presence of liquid nitrogen. Total RNA was prepared using the RNAwiz (AMBION), as described in RNAWIZ procedure. The total RNA was further digested by DNase in RNAWIZ procedure. The total RNA was further digested by DNase I in RNAWIZ procedure. The total RNA was further digested by DNase I in RNAWIZ procedure. The total RNA was further digested by DNase I in RNAWIZ procedure.

Cloning of PI-PLC cDNAs from Lilium daviddi pollen

The full-length cDNA encoding PI-PLC was obtained by a combination of RT-PCR and RACE cloning. Total pollen RNA (5 μg) was used to synthesize the first stand cDNA using SUPERSCRIPT II RT (GIBCO BRL) reverse transcriptase. Two cDNAs fragments were amplified using two primers, 5′-ccagggctgtccaggggatcgc-3′ (upstream) and 5′-actaaacagcggcggcctacaagac-3′ (downstream). The lengths of primers of LdPLCs were about 1.8 kb, respectively. As the control, actin cDNAs were amplified using the same RNA samples in the program of 94°C, 1 min; 94°C, 30 s, 60°C, 30 s, 72°C, 10 min. The primers of actin-exon cDNA were 5′-agaactctggat-
Calcium fluorescent indicator loading and the measurement of [Ca^{2+}]_{cytosol}

Pollen grains were hydrated for 30 min with BK solution which consists of 1 mmol l^{-1} Ca(NO_3)_{2}, 1 mmol l^{-1} KNO_3, 1 mmol l^{-1} MgSO_4, 1 mmol l^{-1} boric acid, 0.5 mol l^{-1} sucrose. The method for loading fluorescent indicator under low-temperature was accorded to Zhang and Rengel (1998). Adding the stock fluo-3AM solution (Molecular Probes, dissolved in DMSO) into pollen grain suspension to a final concentration of 20 μmol l^{-1}. Then pollen grains were incubated in dark at 4°C for 1 h, centrifuged and washed with BK solution twice, then incubated in dark at 25°C for 30 min. A confocal laser scanning microscope (Bio-Rad, MR/A-2) was used to measure calcium distribution and fluorescent intensity. During the scanning period, U-73122 was added in bathing medium to trace cytoplasmic calcium dynamics in pollen cells. The experiment was repeated more than 3 times, in each treatment, data from 20–30 cells were recorded.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

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