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Localization of calcium signaling components in pollen and stigma of sunflower

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Abstract
The intracellular signaling system based on calcium is linked to the external stimuli for mediating a wide range of endogenous responses. The concentration of the free cytosolic calcium ($[Ca^{2+}]_{cyt}$) is central to this calcium-based signaling. In addition to ($[Ca^{2+}]_{cyt}$), other components of this signaling cascade, such as calcium channels, calmodulin and protein kinase also play important role. Present work has reported the spatial localization of these components in mature pollen and receptive stage of stigma. This work is the first report of L-type calcium channels in the membrane of pollen as well as stigmatic papillae. Abundance of intracellular calmodulin in pollen and papillae has been reported. Protein kinase C has been found to be differentially distributed on the pollen coat and in the plasma membrane of papillae. These findings are evident of an active calcium-based signaling mechanism which could be prerequisite for success of initial phase of pollen stigma interaction.

Keywords: calcium channel, calmodulin, protein kinase C, pollen, stigma

1. Introduction

Calcium is one of the important ubiquitous intracellular secondary messengers in plants. Ca^{2+} ions are stored in vacuoles, endoplasmic reticulum, mitochondria and cell wall, and is released whenever required by the cell [1]. It is a versatile signal molecule which regulate several physiological functions, such as growth and differentiation, photomorphogenesis, embryogenesis, hypersensitive responses induced by pathogens, assembling and disassembling of cytoskeleton component. Several external stimuli, such as light and various stress factors, can bring about changes in cellular Ca^{2+} level, which can affect plant growth and development. It is extensively used in living organisms to couple extracellular stimuli to their characteristic intracellular responses to coordinate several endogenous processes [2].

During pollen-pistil interaction Ca^{2+} dynamics in pollen and pistil are important for the complex mechanism that operate between them. The precise functions of Ca^{2+} dynamics in the cells, however, remain unknown. Based on the differential spatial and temporal characteristics of Ca^{2+} distribution in both reproductive tissues, speculations have been made about its role during their interaction [3]. Calcium signaling has also been reported to mediate self-incompatibility responses [4]. Calcium channels are considered to play prominent roles in signal perception and transduction by plant cells. In pollen tube tips Ca^{2+} gradients are essential for its guidance. The loss of polarized pollen tube growth is correlated with the loss of the tip-focused Ca^{2+} gradient [5]. The Ca^{2+} channels in pollen tube tips are involved in the formation of Ca^{2+} gradients by facilitating influx of external Ca^{2+}. They restore basal Ca^{2+} concentrations by extruding it out of the cytosol. Thus, the Ca^{2+} channels are the key components for regulating the Ca^{2+} gradients and are consequently essential for pollen tube guidance [6]. In Arabidopsis, Ca^{2+} influx in papillae of stigma mediated by glutamate receptor-like channel (GLR) leads to rejection of self-pollen [7]. A high apical concentration of intracellular ($[Ca^{2+}]_{cyt}$) in growing pollen tubes is essential for its growth and elongation. This is due to localized Ca^{2+} influx through active Ca^{2+} channels at the pollen tube tip [8].

The receptivity of stigma has been characterized with accumulation of reactive oxygen species (ROS) and nitric oxide (NO) [9]. Both NO and ROS have been implicated in mediating signaling responses in tip growing cells, such as pollen tube [10]. NO acts as Ca^{2+} mobilizing messenger in plants (High apical levels of ROS modulate root hair-tip growth by activating a Ca^{2+}channel which is required to generate tip-focused Ca^{2+} gradient [11]. NO has been reported to mediate part of its effect through the mobilization of free Ca^{2+} via modulation of protein kinase activities and by interacting with ROS [12]. Calmodulin (CaM) acts as a multifunctional Ca^{2+} receptor protein in the intercellular space. Post pollination CaM has been detected in the extracellular matrix of transmitting tissue that surround the tips of growing pollen tubes, suggesting that sufficient apoplastic CaM is needed for pollen germination and tube growth [13]. Phosphorylation cascades are known to be a major part of signaling pathways in plant cells, particularly those involving Ca^{2+}. Protein Kinase-C (PKC; EC 2.7.11.13) is a member of the group of AGC kinases that are involved in controlling the function of other proteins via the phosphorylation of hydroxyl groups of serine and threonine amino acid residues. The PKC enzymes are activated by the increased concentration of diacylglycerol levels (DAGs), Ca^{2+} and phospholipids thereby playing an important role in signal transduction cascades [14].

Recent investigations of pollen and stigma in sunflower have highlighted spatial and temporal distribution of novel biomolecules including glycoprotein, proteases and peroxidases [15]. Earlier work on sunflower in context with pollen-stigma interaction has reported lipidic constituents and associated enzymes [16], accumulation and scavenging of ROS and NO [17]. The calcium signaling aspect remains an unexplored area in context of PSI in sunflower so far. Present work has been carried out to localize the components of calcium signaling machinery, such as calcium channels, calcium-calmodulin complex and protein kinase C in mature viable pollen and the mature receptive stage of stigma. The spatial distribution of these significant components in pollen and stigma has been discussed for their possible signaling role during the initial interactive event between them.

2. Materials and Methods

2.1 Plant material

Present work has been carried out on sunflower (*Helianthus annuus* L. CV Morden), an important oilseed crop. The seeds for raising the crop were procured from National Seeds Corporation, Delhi. Seeds were washed and imbibed in distilled water for 4 h. Imbibed seeds were sown in the garden of Department of Botany, University of Delhi. After attaining reproductive maturity sunflower capitulum were excised at stage 5.3 [18]. At this stage about 30% of the inflorescence was flowering. Pollen grains were harvested immediately after anthesis (Fig. 1a, f) whereas stigmas were harvested 1 d after anthesis (pistillate stage) when it is highly receptive (Fig. 1g).

2.2. Pollen viability test

Pollen viability was determined by treating them with fluorescein diacetate (FDA; 0.01%) [19]. Pollen grains were incubated in 0.01% FDA for 5 min at room temperature (RT) and examined for fluorescence upon excitation at 490 nm and emission at 514 nm, using fluorescence photomicroscope (Axioskop, Zeiss, Germany) fitted with filter no. 9 (excitation filter BP 450-490, beam splitter FT 510 and emission filter LP 520). Viable pollen grains fluoresced bright green (Fig. 1b).

2.3 Localization of Calcium Channels

Sites of calcium channel activity (verapamil-binding sites) were localized in stigma and pollen grains by labeling them with DM-Bodipy verapamil [20]. From a stock solution of 2.6 mM prepared in DMSO, 2 µl was diluted to 1ml with 20% DMSO to get 5.2 µM stock II. Samples were subjected to 1 µM of the fluorescent probe by incubating the material for 30 min in 500 µl of the incubation solution (prepared by diluting 96 µl of stock II of the fluorescent probe to 500 µl using distilled water) at 20°C. The samples were examined for fluorescence (excitation filter BP 450-490, beam splitter FT 510 and emission filter LP 520) using fluorescence photomicroscope (Axioskop, Zeiss, Germany).

2.4 Localization of Membrane-Bound Calcium

Membrane-bound calcium was localized in pollen grain and stigmatic papillae using Chlorotetracycline (CTC) [21]. The squash mount of stigmatic papillae and pollen samples were treated with 0.2 mM solution of CTC. Samples rinsed with 1 mM aqueous solution of EGTA (Ethyleneglycol-bis- (2-amino ethyl ether)- N, N, N, N-tetracetic acid) for 15 minutes before treatment with CTC were used as controls. Samples were examined for fluorescence (excitation filter BP 450-490, beam splitter FT 510 and emission filter LP 520) using a fluorescence photomicroscope (Zeiss, Germany).

2.5 Localization of Calcium -Calmodulin Complex

Calcium-Calmodulin complex (Ca^{+2}-CAM complex) was localized in the stigmatic papillae using Trifluoroperazine (TFP) [21]. Samples were treated with 20 µM solution of TFP for 1-2 min followed by mounting in distilled water. The specificity of TFP was investigated by first incubating the stigmatic papillae in 10 mM solution of EGTA for 15 min and then transferring them into a solution containing 20 µM TFP and 50 µM EGTA. Samples were observed for fluorescence (excitation filter BP 450-490, beam splitter FT 510 and emission filter LP 520) using fluorescence photomicroscope (Axioskop, Zeiss, Germany).

2.6 Localization of Protein Kinase C Activity

Protein kinase C activity was localized in stigmatic papillae and pollen grains by using fim-1 diacetate as fluorescence probe [22]. Stigmatic papillae and pollen grains were incubated with 1 µm fim-1 diacetate for 30 min. After incubation samples were mounted in distilled water and observed for fluorescence (excitation filter BP 450-490, beam splitter FT 510 and emission filter LP 520) using fluorescence photomicroscope (Axioskop, Zeiss, Germany).

All the *in situ* localization experiments were performed in 5-6 samples for each treatment. All the fluorescent images were taken using digital camera (Axiocam, Zeiss, Germany) fitted with fluorescence microscope (Axioskop, Zeiss) and images were processed using Axiovision software (Zeiss, Germany).

3. Results and Discussion

Calcium is a ubiquitous second messenger in the eukaryotic signal transduction cascade. Understanding of these emerging aspects of Ca^{2+} signaling in plants have, however, been started recently. The combined action of Ca^{2+} influx via Ca^{2+} channels and efflux via Ca^{2+} transporters produces Ca^{2+} signatures specific to a process. These channels form the influx or ‘on’ component of Ca^{2+} signaling. Pollen germination is a calcium-dependent process *in vitro*. Normal pollen-tube growth also depends on the optimal concentration of calcium ions in the medium. The involvement of Ca^{2+} in transferring information between the pollen grain and stigma cannot, thus, be ruled out.

3.1 Calcium signaling molecules on pollen

Bodipy-FL-verapamil is a well-known fluorescent probe for localization of L-type calcium channels. Pollen grain fluoresced bright green at certain sites upon treatment with probe (Fig 2a). This fluorescence pattern has been observed in the membrane rather than pollen wall or pollen coat. Thus, confirming the presence of calcium channels in the cell membrane of pollen. Sunflower being a sporophytic self-incompatible exhibit difficulty in *in vitro* pollen germination. Several protocols have been worked out for in vitro pollen germination of sunflower pollen but none resulted in satisfactory pollen germination. Therefore, localization of calcium channels in pollen tube could not be done. Ca^{2+} gradients in the pollen tube tips are essential for pollen tube guidance and that plasma membrane Ca^{2+} channels in membrane of pollen tube tips are core components that regulate Ca^{2+} gradients by mediating and regulating external Ca^{2+} influx. Therefore, Ca^{2+} channels are the core components for pollen tube guidance. Gao *et al*., 2016 reported that Cyclic nucleotide-gated channel 18 (CNGC18) is an essential Ca^{2+} channel in pollen tube tips for pollen tube guidance to ovules in Arabidopsis. Earlier reports of localization of calcium channel activity in pollen tube have highlighted their importance in polarized growth as they take care of gradient of free Ca^{2+} inside pollen tube [23, 24, 25].

Calmodulin (CaM) is a calcium binding protein that gets activated upon binding of Ca^{2+} intracellularly to form Ca^{2+}-CaM complex and participates in cellular function in eukaryotes. These Ca^{2+}-CaM complexes and cytosolic calcium have been found to be uniformly distributed inside pollen (Fig 2 b, c). Moutinho *et al*., 1998 reported uniform distribution of CaM in growing pollen tube except at the apical region (V collar present) which disappears as the tube ceases its growth [26]. It shows that the pollen grain post germination shows the same distribution of CaM as non-germinated pollen except at apical region while the pollen tube is still growing. CaM and actin dynamics have been hypothesized to be involved in regulation of calcium channels [27]. In Arabidopsis, under low calcium concentration Ca^{2+}-free CaM2 bind to CNGC18 which leads to opening of channel, thus allowing entry of Ca^{2+}. When concentration of Ca^{2+} becomes critical then CaM2 binds to the calcium causing its dissociation from the complex. This inactivates the channel and stop the influx of Ca^{2+} [28].

Germinating pollen grains have been reported to contain Ca^{2+}-dependent protein kinases, which have been hypothesized to participate in the Ca^{2+}-mediated signal transduction between pollen and stigma [29, 30]. The PKC family of enzymes transduces the myriad of signals promoting lipid hydrolysis. One class of protein kinase C isozymes is activated by Ca^{2+}, which increases the affinity of the enzyme for phosphatidylserine. PKC Increase Ca^{2+}-stimulated secretion by modulating membrane-attached exocytic machinery [31]. Treatment of pollen with Fim-1 diacetate revealed the presence of protein kinase C enzyme on the surface of pollen grain, which fluoresced golden-yellow (Fig. 2d). The pollen coat of *Brassica napus* contains, a 24 kDa protein kinase whereas two protein kinases (31 and 35 kDa) have been reported in the pollen coat proteome of *Arabidopsis thaliana* [32]. Consistent with earlier reports, a protein kinase i.e. PKC is localized on the surface of pollen grains. Present work has employed fluorescent probe for the localization of PKC activity whereas earlier workers have used molecular methods for characterization of protein kinases.

3.2 Calcium signaling molecule in stigma

Calcium has been reported to be present on the surface of both wet and dry type of stigma. The membrane of the stigmatic papillae showed presence of calcium channels (Fig. 3a). This is the first report of calcium channel localization in stigma. Stigmatic papillae show a general distribution of Ca^{+2}-CAM complex throughout the cell (Fig. 3b). These events indicate the existence of calcium-dependent processes in stigma. CTC had been used for localization of CTC-Ca^{2+} complex in *Primula officinalis* (having dry type stigma) and *Ruscus aculeatus* (having wet type stigma) [33, 34]. The stigma of sunflower is of semi-dry type as there are no apparent exudate present during the receptive period. Instead scanty secretions are present at the base of the stigmatic papillae which are not visible. In sunflower stigma, the papillae fluoresced strongly at certain places indicating the presence of membrane-bound calcium in the cytoplasm near the wall (Fig 3c). The CTC-Ca^{2+} fluorescence was also observed in the form of thin layer probably pellicle that surrounds the cell wall. This indicates that calcium ions are bound to proteinaceous pellicle in the same way as membranes. Washing of stigma with 1mM aqueous solution of CTC inhibited the CTC fluorescence. However, the inhibition of CTC fluorescence is a reversible process *in situ* as papillae resume fluorescence after few hours of calcium ion chelation with EGTA (data not shown). These observations are concomitant with the observations in *Primula officinalis* [35]. Studies in *Petunia hybrida* have shown that in unpollinated stigma the level of CTC-Ca^{2+} fluorescence decreases from stigma to the base of the style and again strong fluorescence in placenta and ovule [36]. Upon pollination this fluorescence pattern showed reversal with base of style showing maximum fluorescence.

In stigma, receptor-like protein kinase (encoded by SRK gene) has been localized to the plasma membrane of the papillae in *Brassica* that cover the surface of stigma [37]. In accordance with the previous reports, present work on protein kinase has also demonstrated their presence on the surface of stigmatic papillae. The surface of the stigmatic papillae fluoresces golden-yellow, indicating the presence of protein kinase C in the membrane of stigmatic papillae (Fig. 3d). It has been proposed that receptor-like protein kinase function in SI signal transduction pathway since various *B. oleracea* and *B. campestris* mutants or *B. napus* lines that are presumably deficient in the recognition of self-pollen, have severely reduced SRK activity [38,39].

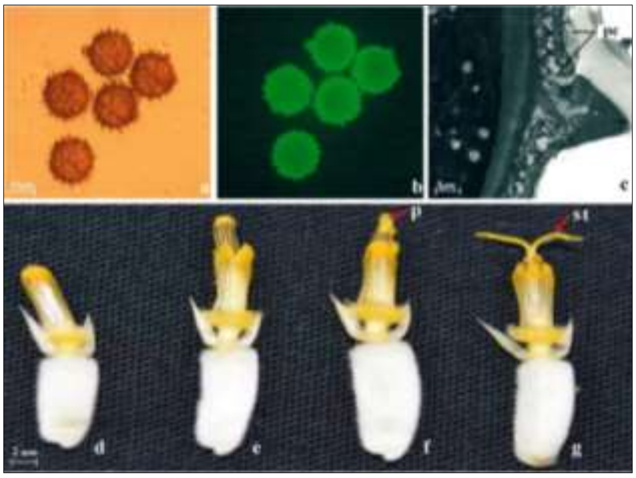

Fig 1: (a) Sunflower pollen grains as viewed under light microscope at 200X, (b) As viewed under fluorescence microscope upon excitation at 490 nm following treatment with fluorescein diacetate at 200X, (c) Transmission electron micrograph of pollen showing details of spinular region at 4,600X (adapted from Shakya, 2008) [40], (d-g) Different developmental stages of sunflower ray florets showing (f) anther dehiscence and (g) emergence of receptive stigma. Pc- pollen coat, s- spinule, p-pollen, st- stigma.

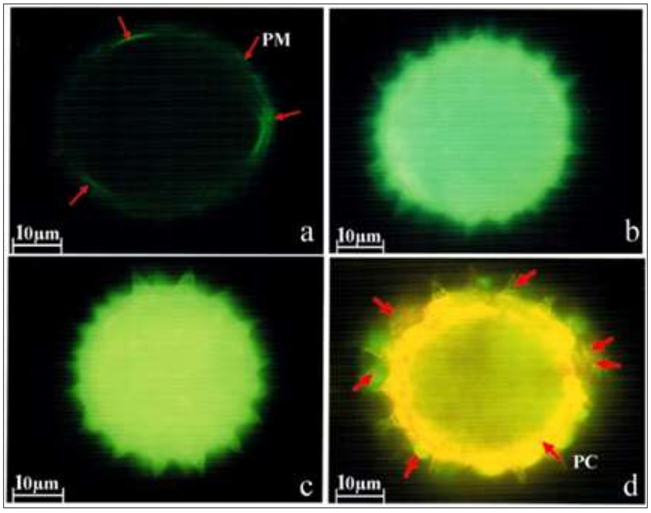

Fig 2: (a) Localization of calcium channel sites (b) Calcium-calmodulin complex (c) cytosolic calcium ions (d) and calcium-dependent protein kinase C (PKC) on pollen grain of sunflower. PM- plasma membrane, PC- pollen coat.

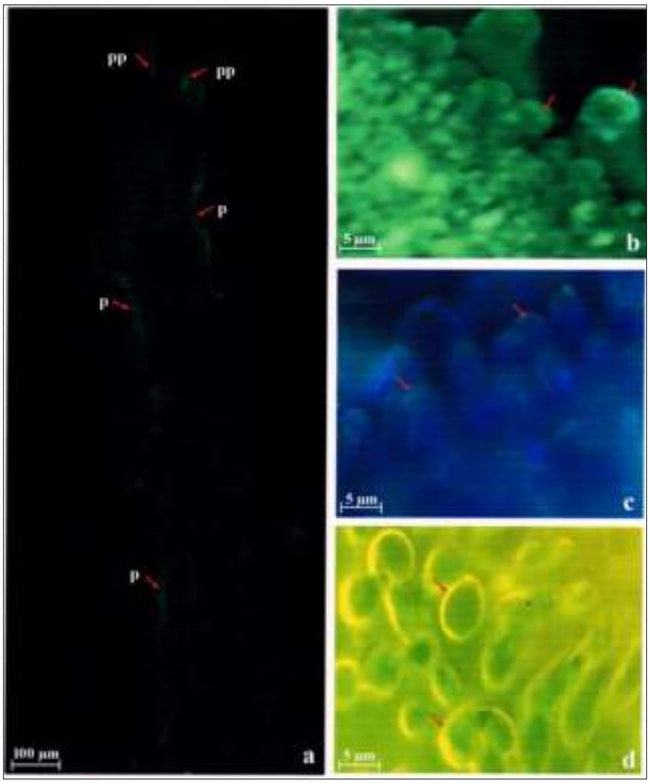

Fig 3: Localization of (a) calcium channel sites, (b) Calcium-calmodulin complex, (c) cytosolic calcium ions, (d) and calcium dependent protein kinase C (PKC) on stigmatic papillae of receptive stigma of sunflower. Arrows show localization sites.

4. Conclusions

Present work on localization of calcium signaling molecules in pollen and stigma of sunflower has provided a foundation for the exploring the calcium signal transduction research in the pollen-pistil interaction studies in sunflower. Presence of calcium channels, calmodulin and protein kinase C indicates that calcium is likely to be playing a pivotal role in various events that take place during the initial phase of this interactive process. Further studies are required to explore how calcium affects interaction between pollen stigma during initial and later stages. Difficulty in *in vitro* pollen germination of sunflower has posed challenges to study the calcium dynamics in growing pollen tube. Therefore, future investigations should focus on *in situ* dynamics of Ca^{2+} distribution i.e. gradient in pollen tube post pollen germination on the surface of stigma following compatible and incompatible pollination. Studies in calcium signal transduction pathways can be later correlated for their possible cross-talk with ROS and NO signaling mechanisms which have been shown to crucial during initial cellular recognition between pollen and stigma.

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