In situ Localization of Ca^{2+} in Pollen Grains and Pollen tubes of *Vinca rosea* using the Antimonate Precipitation Technique

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Using the antimonate precipitation technique Ca^{2+} has been localized in the mature pollen grains and germinating pollen tubes of *Vinca rosea in vivo*. Before the emergence of the pollem tube, in the pollen grains high Ca^{2+} containing areas were found to be associated with the cytoplamic membrane adjacent to the intine. The Ca^{2+} present in the cytoplasm could be noted as numerous black Ca^{2+} – antimonate precipitates. With the onset of pollen germination on the receptive surface of the papillate stigma, a rapid accumulation of high Ca^{2+} concentration at the tip site of the pollen tubes was recorded. The inner wall of the germinating pollen tube tips contained uniform Ca^{2+} positive areas whereas, the older areas of the inner wall towards the pollen had no appreciable amount of Ca^{2+} positive sites associated with it.

The Ca^{2+}–antimonate precipitates were however, not present uniformely on the outer surface of the pollen tubes present in the stigmatic tissue. In such of those pollen tubes that had penetrated the stigma, a transparent region of the tube tips resembling the 'cap–block' did not contain any Ca^{2+} – precipitates.

Introduction

It is now being widely recognized that Ca^{2+} plays an important role in the regulation of various eukaryotic cell functions, either directly, or indirectly, via the "second messenger" system involving Ca^{2+} –dependent regulatory proteins like calmodulin (1–6). In this laboratory, we have been interested in elucidating the possible roles of Ca^{2+} and calmodulin in the reproductive physiology of higher plants. The results from our laboratory (7) have demonstrated that purified bovine brain calmodulin can stimulate the pollen *Vinca rosea* to germinate more rapidly by reducing the length of the lag–period before pollen tube emergence. It has also been suggested (7) that calmodulin induced stimulation of pollen germination and tube elongation could occur by regulation/mobilization of endogenous Ca^{2+} levels in a Calmodulin–dependent process. We have recently found (unpublished data) that various calmodulin–antagonistic druge like chlorpromazine (CPZ), trifluoperazine (TFP), 2–Trifluoromethyl–10H–10–(3'aminopropyl) phenothiazine (TAPP), N–(6–Aminohexyl)–5–chloro–1–naphthalenesulfonamide (W–7) and N–(6–Aminohexyl)–1–naphthalenesulfonamide (W–5) inhibit pollen germination and tube elongation in *Vinca rosea*. These findings indirectly

suggest that the events underlying pollen germination and tube growth are possibly modulated by calcium-calmodulin complex. In this paper we examine the Ca^{2+}-distribution changes in the pollen of *Vinca rosea* before and after pollen tube growth *in vivo* employing the antimonate procipitation technique developed by Slocum and Roux (8) to localize Ca^{2+} under carefully defined experimental conditions.

Materals and Methods

Flowers of *Vinca rosea* (Catharanthus roseus L.) growing in a farm of the department were collected before and after anthesis. The stigma exudes a sticky substance before anthesis. The stigma tissue was sampled at 5, 10, 15 and 30 min and at 1, 2 and 3 h before and after pollination. The pollinated and unpollinated stigma tissue was directly fixed in accordingly as described by Slocum and Roux (8) using Karnovsky's fixative(9)containing 2% glutaraldehide 2.5% formaldehyde /2% potassium antimonate Ksb $(OH)_6$ in 0.1M potassium phosphate buffer, pH 7.6 for 6 h at 21°C in total darkness. Samples were carefully washed at least three times in phosphate buffer pH 8.0, without antimonate. Individual stigma and their sectors were carefully dissected in wash buffer under the microscope. The sectors of the stigma tissue having the pollen grain and/or the pollen tubes were isolated using a sharp needl and a cover slip was gently placed on them. Following antimonate precipitation some of the pollen grains were placed in a drop of a hypotonic sodium chloride (NaCl) solution subsequent to washing in buffer solution. This post treatment of the stained pollen caused partial plasmolysis in them. The plasmolyzed pollen preperations were then observed microscopically. For cotrol, the stigma tissue were fixed as described above, however the fixative did not contain any potassium antimonate in it. All specimans including those of the control were photographed under standard bright field illumination using 'Meiopta' light microscope.

Results and Discussion

In the mature pollen the inner surface of the cell has Ca^{2+}-precipitates associated with it (Fig. 1), however, following partial plasmolysis of the stained pollen it is found that most of the precipitated Ca^{2+} is confined in the cytoplasmic membrane of the pollen. A number of black Ca^{2+}-antimonate precipitates are present in the pollen cytoplasm (Fig. 1). The stigma tissue sampled after 1h following anthesis had a number of pollen on the surface that had just begun to germinate however, the pollen tubes had not penetrated the stigma surface. In such of the pollen tubes present on the papillate surface of the stigma, most of the antimonate precipitated Ca^{2+} is present in the tip area and the cytoplasm of the tube away from the tip had very few precipitates (Fig. 2). Many of the germinating pollen on the

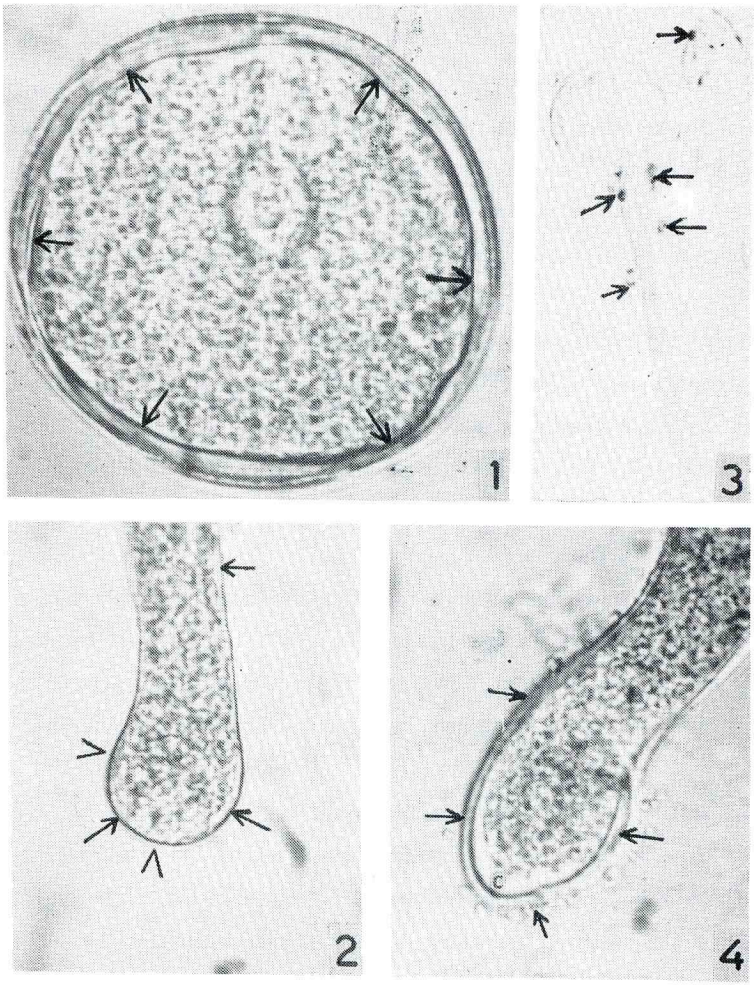

Fig. 1 Antimonate precipitaion of Ca^{2+} in the mature pollen. Note the high Ca^{2+} staining areas associated with the cytoplasmic membrane (arrows) adjacent to the intine and also the black Ca^{2+} −antimonate precipitates in the cytoplasm. × 500.

Fig. 2 Note the relatively more number of Ca^{2+}−precipitates near the tip area of the pollen tube present on the surface of the stigma that was sampled after anthesis. The outer wall of these pollen tubes (darts) do not have any precipitated Ca^{2+} whereas, the inner membrane areas (arrows) have distinct Ca^{2+}-antimonate precipitates particularly at the tip site. Note the low Ca^{2+} content of the membrant present away from the tip. × 450

Fig. 3 Note the general absence of distinct Ca^{2+}-precipitates in the pollen grain and its tube, of the control sample that was incubated in the solution devoid of potassium antimonate. The black areas (arrows) present around the germnating pollen are those of the stigmatic debris that could not be washed off by the buffer. This sample was isolated from the stigmatic tissue 2 h after anthesis. ×150

Fig. 4 Ca^{2+}—antimonate precipitates in the pollen tube tip present inside the solid stigma tissue 2 h after pollination. No precipitates are present in cap block (c) like area. Note the presence of black Ca^{2+} positive nature of the outer wall (arrows) of the tube. ×450

surface of the stigma had slightly bulged tips and the inner membrane areas of these pollen tubes invariably contained Ca^{2+}. Similar concentration gradient of Ca^{2+} has been demonstrated *in vitro* growing pollen tubes of *Lilium* (10)' using the fluorescent calcium indicator chlorotetracycline. Reiss *et al* (11), further confirmed the tip to base Ca^{2+} gradient in the pollen tubes, using proton-induced X-ray emission. The results in the present study clearly show that Ca^{2+} gradients exist in the *in vivo* (Fig. 2) germinating pollen tubes and that these gradients are almost identical to those observed *in vitro* growing pollen tubes as recorded *Lilium* (10).

In vivo germinating pollen samples incubated in the reaction mixture without potassium antimonate do not contain any black Ca^{2+}-antimonate precitates in them (Fig.3), this invariably indicates that the black precipitates observed in the samples (Figs. 1,2 and 4) are formed in the presence of antimonate anions in the incubation medium. Furthermore, because the antimonate anion has a relatively high affinity for Ca^{2+} (12) it can complex most forms of Ca^{2+} (free and bound) within the cell (8).

The pollen tubes that had penetrated the stigma surface invariably had a small transparent area at their tips resembling the 'cap block' (Fig. 4). This cap block like area was devoid of Ca^{2+}-antimonate precipitates, however, the inner membrane area of the extreme tips adjacent to the cap block like area contained significantly high Ca^{2+} positive antimonate precipitates (see Fig. 4).

The outer wall of these pollen tubes stained positively for Ca^{2+}, this is in contrast to the situation observed of the pollen tubes present on the surface of the stigma (Fig. 2) Wherein, the outer wall of the pollen tubes do not show any precipitated Ca^{2+}. The high Ca^{2+} associated with the outer wall of the pollen tubes which present in the stigmatic tissue (Fig. 4), is most probably derived from the surrounding cells of the stigma. The high influx of Ca^{2+} into the stigma tissue before anthesis (data communicated elsewhere) indirectly supports this notion.

As it has been suggested by Iwanami (13) that the cap block appears in the growing pollen tube tipe and that it disappears when the tube ceases to grow, we are inclined to consider that the antimonate precipitation of Ca^{2+} in the pollen tubes (present in the stigmatic tissue Fig. 4) was at a stage of their active growth *in vivo*.

The results of the antimonate precipitation technique employed in the present investigation to localize Ca^{2+} in the pollen and germinating pollen appears to be semiquantitative in nature. Studies using permeant fluorescent Ca^{2+} indicators like quin-2(14), fura-2(15) and the polyether antibiotic (16) ionophore A23187 could provide exciting new information about Ca^{2+} distribution and mobilization in the reproductive systems of higher plants. Nevertheless, at the light microscopic level the antimonate precipitation technique can provide useful information on the relative concentrations of subcellular Ca^{2+} in different types of plant cells.

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