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Involvement of Ca^{2+} in 1-Aminocyclopropane-1-Carboxylic Acid-Stimulated Pollen Germination

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Abstract. The role of 1-aminocyclopropane-1-carboxylic acid (ACC) in pollen germination was investigated in several plant species. It was found that ACC stimulated in vitro pollen germination in all five species of plants tested. EGTA and phenothiazine inhibited the increase in the germination rate induced by ACC. Free Ca^{2+} levels in the cytosol ($[Ca^{2+}]_{cyt}$) in ungerminated and germinated pollen were 136 and 287 nM, respectively. Adding 0.25 mM ACC to the germination medium increased the $[Ca^{2+}]_{cyt}$ in germinated pollen up to 450 nM. When pollen was treated with both 0.25 mM ACC and 3.6 μM inositol 1,4,5-trisphosphate, the $[Ca^{2+}]_{cyt}$ increased to 850 nM, and pollen germination was also stimulated. In the presence of Li^+, an inhibitor of inositol monophosphatase, the $[Ca^{2+}]_{cyt}$ was reduced to 155 nM, and the ACC-stimulated pollen germination was inhibited. The data provided evidence for the involvement of Ca^{2+} as a messenger in the stimulative effect of ACC on pollen germination.

Key Words. ACC—Calcium—Inositol 1,4,5-triphosphate—Pollen germination—Signal transduction

1-Aminocyclopropane-1-carboxylic acid (ACC) is the immediate precursor of the phytohormone ethylene. It has been shown that ACC is present in pollen grains, and pollination may increase the production of ethylene and thereby accelerate flower senescence (Whitehead et al. 1983). When exogenous ACC was applied to the stigma of a nonpollinated flower, it caused an increase in ethylene production similar to that induced by pollination, resulting in a rapid senescence of the flower from ovary to corolla (Reid et al. 1992). The high levels of ACC and ethylene production occurring in stigma, especially after pollination, imply that ACC may participate in the process of pollen germination (Piskornik 1986, Piskornik et al. 1989). It has been suggested that ACC is translocated in the style through the ovary to the corolla, where it induces its early senescence effect. Therefore, ACC may function as a second transmissible signal that coordinates postpollination development in diverse floral organs (O'Neill et al. 1993). Unfortunately, the mechanism of the stimulative effect of ACC on flower senescence via ethylene is still not known.

The necessity of Ca^{2+} for pollen germination and polarized growth of pollen tube tip is well documented (Heslop-Harrison 1987, Tirlapur and Cresti 1992, Miller et al. 1992). Calmodulin (CaM) has also been shown to play a role during pollen germination and pollen tube growth. In addition, phospholipase C activity has been reported to occur in pollen (Drøbak 1992). The level of inositol 1,4,5-trisphosphate (IP_3) and the turnover of several precursors of IP_3 are regulated by physiological stimuli such as light, osmotic stress, and phytohormones (Cote and Crain 1993, Ettlinger and Lehle 1988). There is no direct evidence for coupling of stimuli, e.g. phytohormones, and physiological response.

Hence, the primary aim of this paper was to investigate the possible role of ACC in pollen germination. Using the calcium indicator fura-2, we provide evidence that ACC regulates cytosolic Ca^{2+} concentration via ethylene during pollen germination. These studies establish a connection between pollen germination and hormone stimulus in signal transduction.

Materials and Methods

Pollen Germination

Fresh pollen grains of five species of plants, *Amelanchine sinica* (Shneid) Chun, *Cercis chinensis* Bungle, *Prunus triloba* Lindal, *Sophora japonica* L., and *Cucumis sativus* L. cv. Bianjing 4, were

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; CaM, calmodulin; IP_3, inositol 1,4,5-trisphosphate; GM, germination medium.

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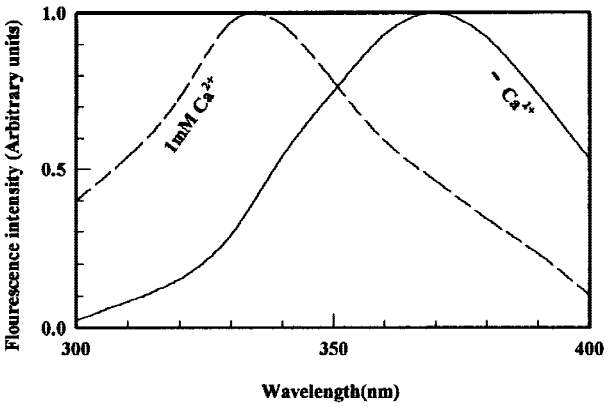

Fig. 1. Excitation spectra for 1 μM fura-2-AM in pollen at saturating Ca^{2+} (1 mM) and zero Ca^{2+} (5 mM EGTA added). Emission was measured at 450 nm. *Broken line,* saturating Ca^{2+}; *solid line,* no Ca^{2+}.

collected from open flowers and cultured at 24°C ± 1°C in germination medium (GM), which consisted of 10% (w/v) sucrose, 100 ppm of boric acid, 100 mM KCl. The following treatments were employed: GM + 0.25 mM ACC; GM + 0.25 mM ACC + 0.5 mM EGTA; GM + 0.25 mM ACC + 0.5 mM phenothiazine (Merck); GM + 0.25 mM ACC + 3.6 μM IP_3 (DojinDo Co., Japan); or 1 mM LiCl. The percentage of pollen germination was measured after 30, 60, 120, and 180 min of incubation.

Fura-2 Application and Fluorescence Measurement

Fura-2 was applied by adding fura-2-AM to a final concentration of 1 μM and 0.03% Triton X-100 to the GM, adjusting the pH to 7.2 by KOH or HCl, and finally incubating the pollen grains for 30 min at 30°C with continuous shaking.

After application of fura-2, pollen grains were sedimented and resuspended twice in incubation medium without dye and then picked up carefully by tweezers and transferred onto a slide. The slide was examined under microscope to ensure that only one pollen grain was loaded in the light path of the fluorescence measurement. Subsequently, fura-2 fluorescence intensity of an individual pollen grain was measured using a fluorescence spectrophotometer (Spex F212 Fluorolog). The fluorescence intensities were recorded at 450 nm under excitation wavelengths of 340 and 370 nm.

Ca^{2+} Calibration

Ca^{2+} was calibrated according to Grynkiewicz et al. (1985) with some modifications. All calcium ions within the pollen were bound to 5 mM EGTA (Kanto Co.), and a calibration curve was constructed for an individual pollen grain. Fig. 1 shows the excitation spectra for 1 μM fura-2-AM in a pollen grain in the presence of 1 mM Ca^{2+} or without Ca^{2+} (bound to 5 mM EGTA). The fluorescence excitation spectrum of the Ca^{2+}-sensitive form of the dye shifts to shorter wavelengths when the dye binds Ca^{2+}. To take full advantage of the wavelength shift induced by Ca^{2+} bound to fura-2, it was necessary to measure first the autofluorescence of pollen. This value was subtracted from the spectra of the dye at the two wavelengths. The R_{max} (at saturated Ca^{2+}) and R_{min} (at zero Ca^{2+}) were calculated from the spectra.

In Mn^{2+} quenching experiments, Mn^{2+} was added to GM in a final concentration of 300 μM.

Results

Effect of ACC and Ethephon on Pollen Germination

A significant increase in pollen germination as well as in tube growth (data not presented) occurred after treatment with 0.25 mM ACC and ethephon (Table 1 and Figs. 2 and 3). The germination percentage of fresh pollen of *C. sativus* and *S. japonica,* for example, was increased by ACC from 53 to 63% and from 10.5 to 29% compared with the control after 30 min of incubation, respectively (Figs. 2 and 3). The promotive effect of ACC occurred later than that of ethephon (Table 1).

Effect of EGTA and Phenothiazine on ACC-Stimulated Pollen Germination

To investigate the stimulative effect of ACC, pollen grains were treated with 0.5 mM EGTA or 0.5 mM phenothiazine in presence of 0.25 mM ACC. The germination percentage of *S. japonica* and *C. sativus* pollen was significantly suppressed by the two compounds (Figs. 2 and 3). Compared with the ACC treatment only, for example, the pollen germination of *C. sativus* in the presence of EGTA and phenothiazine was decreased by 35 and 87.6%, respectively, after 60 min of incubation (Fig. 2).

Effect of IP_3 and LiCl on ACC-Stimulated Pollen Germination

The effect of IP_3 and LiCl on ACC-stimulated pollen germination was examined by using fresh pollen of *C. sativus.* IP_3 increased somewhat the stimulative effect of ACC at both incubation periods (Fig. 4). On the other hand, Li^+, an inhibitor of phospholipase C, significantly blocked the stimulative effect of ACC on pollen germination.

Effect of ACC and IP_3 on Changes of $[Ca^{2+}]_{cyt}$ during Pollen Germination

$[Ca^{2+}]_{cyt}$ in pollen was measured by using fura-2. To examine whether pollen possessed the hydrolytic activity of fura-2-AM, we tested the appearance of a Mn^{2+}-sensitive fluorescence signal in pollen. As Sage et al. (1989) have shown previously, Mn^{2+} does not quench the fluorescence of the fura-2-AM but does quench that of fura-2. Fig. 5 shows the response of the fluorescence signal upon addition of 300 μM Mn^{2+}. When the GM was

Table 1. Effect of ACC and ethephon on pollen germination of several higher plant species.

Time (min)	*A. sinica* (Shneid) Chun			*C. chinensis* Bungle			*P. triloba* Lindal		
	1[a]	2	3	1	2	3	1	2	3
0[b]	24 ± 2.0	23 ± 1.5	27 ± 0.5	17 ± 4.5	17 ± 6.0	12 ± 1.0	18 ± 1.0	15 ± 2.0	16 ± 1.0
60	35 ± 1.5	51 ± 2.0	55 ± 6.0	46 ± 2.6	43 ± 2.4	53 ± 2.8	41 ± 2.0	50 ± 0.5	57 ± 1.4
120	60 ± 1.0	62 ± 2.8	73 ± 0.5	63 ± 2.5	77 ± 3.0	78 ± 3.0	66 ± 2.4	85 ± 2.0	86 ± 3.3

Note. Results are indicated as % pollen germination. Each entry represents the mean ± S.E. of 18 determinations.
[a] 1, GM, (10% sucrose, 100 ppm boric acid, and 100 mM KCl); 2, GM + 0.25 mM, ACC; 3, GM + 0.3 mM ethephon.
[b] These are initial values of treatment with ACC and ethephon, after 30 min of incubation in GM.

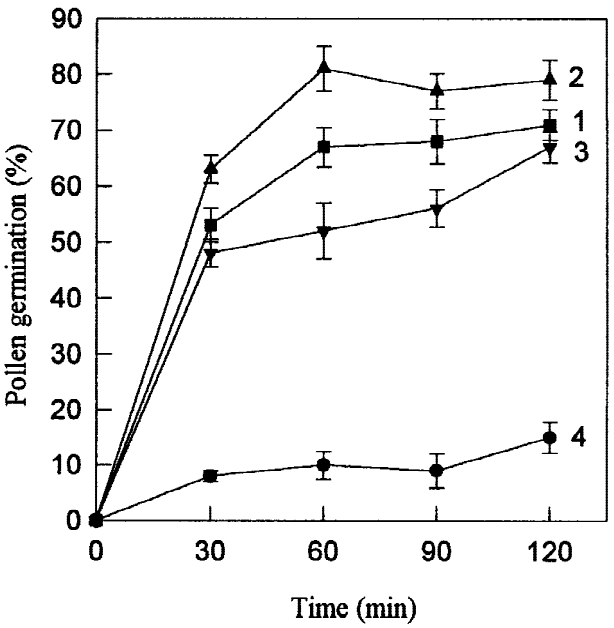

Fig. 2. Effect of EGTA and phenothiazine on ACC-stimulated germination of *C. sativus* L. pollen. *1,* GM; *2,* GM + 0.25 mM ACC; *3,* GM + 0.25 mM ACC + 0.5 mM EGTA; *4,* GM + 0.25 mM ACC + 0.5 mM phenothiazine. Data are the mean ± S.E. of 18 determinations.

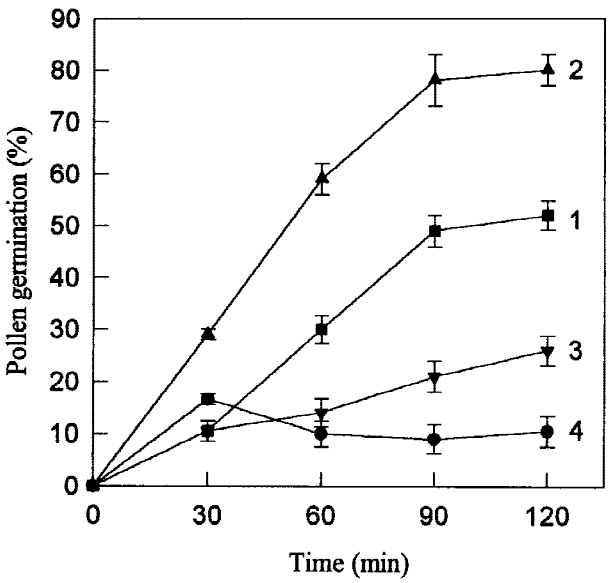

Fig. 3. Effect of EGTA and phenothiazine on ACC-stimulated germination of *S. japonica* L. pollen. Details are as in Fig. 2.

kept free of Ca^{2+} by the presence of 5 mM EGTA, the signal was quenched by Mn^{2+}. The results in Fig. 5 demonstrate that deesterification of fura-2-AM occurs within the pollen grains because the Mn^{2+}-induced quenching shows a marked decrease in the fluorescence intensity at 340 nm.

We found that $[Ca^{2+}]_{cyt}$ of ungerminated and germinated (60 min) pollen were 136 and 287 nM, respectively (Fig. 6). $[Ca^{2+}]_{cyt}$ in germinated pollen increased to 450 nM in the presence of ACC. Similar results were observed for *S. japonica* (data not shown).

Adding of 3.6 μM IP_3 to the medium caused a further increase in $[Ca^{2+}]_{cyt}$, which reached 850 nM. Statistical analysis indicated that the increased percentage of pollen germination was predominantly correlated with the changes in $[Ca^{2+}]_{cyt}$ ($r = 0.96$). However, when Li^+, which inhibits the production of IP_3, was added to the medium, the stimulative effect of ACC on $[Ca^{2+}]_{cyt}$ was blocked.

Discussion

It has been reported that ethylene and ACC may stimulate the germination of pollen grains in vitro (Reid et al. 1992), but their effect varies among different species (Piskornik et al. 1989). We have examined five species in which ACC promotes pollen germination (Table 1 and Figs. 2 and 3). The effect of ethephon on pollen germination was similar to that of ACC. The promotive effect of exogenous ACC on pollen germination indicates that there is a high ability to convert ACC to ethylene in the pollen. To determine the mechanism of the promotive effect of ACC via ethylene, we have tested the effect of EGTA (a Ca^{2+} chelator agent) and phenothiazine (a CaM antagonist) on pollen germination. We observed that both compounds inhibited the stimulation of pollen ger-

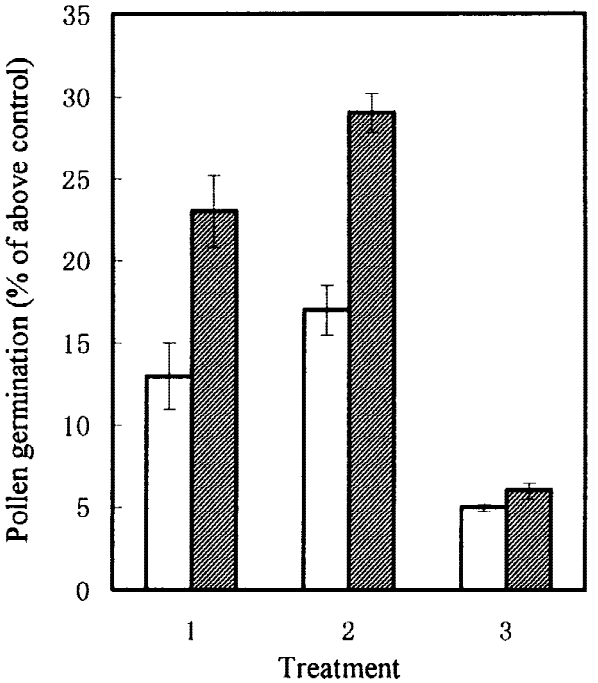

Fig. 4. Effect of IP_3 and LiCl on ACC-stimulated germination of *C. sativus* L. pollen. Pollen grains were cultured for 30 (*clear bars*) and 60 min (*hatched bars*). *1,* GM + 0.25 mM ACC; *2,* GM + 0.25 mM ACC + 3.6 μM IP_3; *3,* GM + 0.25 mM ACC + 1 mM LiCl. The percentage of pollen germination in control at 30 and 60 min was 49 and 57%, respectively.

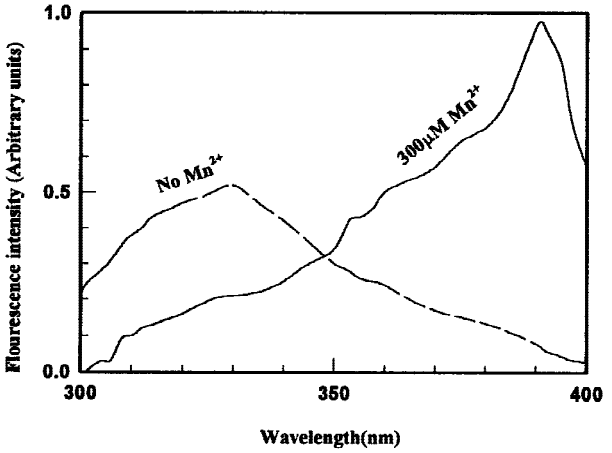

Fig. 5. Excitation spectra for Mn^{2+}-induced quenching of the fluorescence signal in fura-2-AM-loaded pollen. Fura-2-AM-loaded pollen was incubated in GM (10% sucrose + 100 ppm of boric acid + 100 mM KCl) and 300 μM Mn^{2+}. *Broken line,* no Mn^{2+}; *solid line,* 300 μM Mn^{2+}.

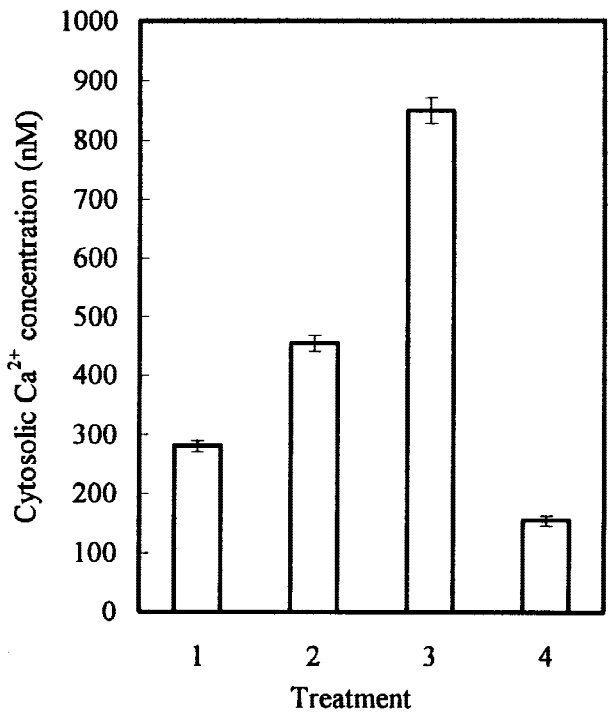

Fig. 6. Effect of ACC, IP_3, and LiCl on $[Ca^{2+}]_{cyt}$ in germinated pollen of *C. sativus* after 60 min of incubation. *1,* GM; *2,* GM + 0.25 mM ACC; *3,* GM + 0.25 mM ACC + 3.6 μM IP_3; *4,* GM + 0.25 mM ACC + 1 mM LiCl. $[Ca^{2+}]_{cyt}$ in ungerminated pollen was 136 nM.

mination by ACC (Figs. 2 and 3). These findings suggest indirectly that the mechanism of ACC-stimulated pollen germination is probably related to the Ca^{2+} messenger system. With the onset of germination, relatively higher amounts of Ca^{2+} and CaM were found toward the aperture through which the pollen tube would emerge (Tirlapur and Cresti 1992). The technique employing a fluorescence probe, fura-2, enabled us to measure Ca^{2+} concentration in a living cell (Song et al. 1997). Our observations of $[Ca^{2+}]_{cyt}$ in pollen using fura-2-AM showed that the $[Ca^{2+}]_{cyt}$ in ungerminated and germinated pollen was 136 and 280 nM, respectively (Fig. 6). At the same time, the $[Ca^{2+}]_{cyt}$ of germinated pollen treated with ACC was about threefold higher than that in ungerminated pollen and also higher than that in germinated control pollen. Similar results were observed during germination of *S. japonica* pollen. Our results on the effect of Ca^{2+} on pollen germination are in agreement with those of Tirlapur and Cresti (1992), who showed that digitization of the fluorescence microscope image of chlortetracycline and fluphenazine-fluorescence emission can reveal a high concentration of membrane-associated Ca^{2+} in apertures through which pollen tubes emerge. The data suggest that the increase in $[Ca^{2+}]_{cyt}$ is a key factor for pollen germination. ACC may increase the Ca^{2+} concentration in pollen rapidly, hence it also promotes the germination of pollen.

Most of the components of phosphoinositide system have been found in plant cells, and a considerable num-

ber of reports point to IP_3-induced Ca^{2+} release from intracellular stores and the involvement of the phosphoinositide in plant development (Cote and Crain 1993). Exogenous IP_3 in the incubation medium enhanced somewhat the ACC-stimulated pollen germination (Fig. 4). In contrast, Li^+, which blocks the turnover of inositol phospholipid and inhibits IP_3 production, not only inhibited the stimulative effect of ACC on the germination of pollen, but also decreased the ACC-induced increase in $[Ca^{2+}]_{cyt}$ in pollen during germination (Fig. 6). These results seem to indicate that the stimulating effect of ACC on pollen germination is probably related to the inositol phospholipid signal system. Although a definite proof for increase of endogenous levels of IP_3 in pollen is still absent, it has been shown recently, by using the patch-clamp technique, that IP_3 regulates the influxes of Ca^{2+} into the cytosol of guard cells (Gilroy et al. 1990). Therefore, the finding that the addition of exogenous IP_3 and ACC together has an additive effect on pollen germination and $[Ca^{2+}]_{cyt}$ also supports the idea of the involvement of $[Ca^{2+}]_{cyt}$ in ACC-stimulated pollen germination.

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