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Ca^{2+} Effect on Conversion of Exogenous 1-Aminocyclopropane-1-Carboxylic Acid to Ethylene in *Vigna radiata* Protoplasts

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The possibility that 1-aminocyclopropane-1-carboxylic acid (ACC)-uptake may be dependent on the H^+-gradient established across the plsma membrane was tested in protoplasts isolated from 2.5 day old mungbean hypocotyls. The ACC-induced ethylene production was inhibited when the H^+-gradient was collapsed by the treatment with carbonycyamide-*p*-trifluro-methoxy-phenylhydrazone (FCCP). Moreover, the treatment with *o*-vanadate, a specific inhibitor of plasma membrane H^+-ATPase, caused the inhibition of ethylene production. The ACC-induced ethylene production was inhibited by the treatment with verapamil (Ca^{2+}-channel blocker), or ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) (Ca^{2+}-chelator). In contrast, the ethylene production was stimulated by the application of A23187 (Ca^{2+} ionophore). The inhibitory effect of EGTA in the ethylene production was magnified in the presence of A23187. From these results, we suggest that the external Ca^{2+} influx to the cytosol resulted in the stimulation of ACC oxidase activity after ACC-uptake resulting from a H^+-gradient across the plasma membrane.

Key words: ACC-uptake, H^+-gradient, Ca^{2+}, ACC-induced ethylene production, ACC oxidase

The conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene, the final step of ethylene biosynthesis, is stimulated by Ca^{2+} (Evensen, 1984). He explained the effect of Ca^{2+} on the ethylene production as follows; the ethylene production from ACC is a membrane associated step, and Ca^{2+} stabilizes the membrane structure and maintains the membrane integrity. Therefore, Ca^{2+} enhances membrane-associated ethylene biosynthesis. In addition, the conversion of ACC to ethylene by ACC oxidase is associated with the membrane (Guy and Kende, 1984).

However, this hypothesis has been challenged. According to the purification of ACC oxidase (Smith *et al.*, 1992) and the analysis of DNA sequence encoding ACC oxidase (Dong *et al.*, 1992; Smith *et al.*, 1992), ACC oxidase may be located in the cytosol (Ayub *et al.*, 1993; Christoffersen *et al.*, 1993). Therefore, the Ca^{2+} action in the conversion of ACC to ethylene should be examined in the other respects.

The exogenous ACC is transported into the cytosol through the H^+/amino acid-cotransporter in the plasma membrane like the other amino acids (Bush and Langston-Unkefer, 1988). On the basis of this fact, we supposed that transported H^+ into the cytosol after ACC-uptake could depolarize the membrane potential in the plasma membrane. Since the depolarization of the membrane potential causes to open the Ca^{2+}-channel in the plasma membrane (Starrach *et al.*, 1984), the concentration of cytosolic Ca^{2+} could be enhanced by the influx of external Ca^{2+} after ACC-uptake. The enhanced Ca^{2+} might affect the ACC oxidase activity in the cytosol.

Based on these results, this reserch focused on the study of the following possibility; The stimulation of the conversion of ACC to ethylene by Ca^{2+} *in vitro* is not resulted from the stabilization of the me-

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mbrane structure, but caused by the stimulation of the ACC oxidase activity according to the increase of cytosolic Ca^{2+} concentration after influx of the exogenous ACC.

In order to investigate this possibility, we isolated the protoplasts from the 2.5 days old mungbean hypocotyls to use in this experiment. And we examined 1) the dependence of the exogenous ACC-uptake on the H^+-gradient established in the plasma membrane and 2) the pattern of the ACC-induced ethylene production in the presence of compounds which alter Ca^{2+} influx.

MATERIALS AND METHODS

Plant material

Mungbean (*Vigna radiata* W. cv. Sunhwa) seeds were obtained from Crop Experiment Station (Suwon). The seeds were soaked overnight in tap water and germinated on the agar plate containing 10^{-2} M $CaCl_2$ for 2.5 days at 27± 1℃ in complete darkness. The humidity was kept over 90%. For the reduction of ethylene effects on the seedlings growth, 4×10^{-5} M $KMnO_4$ was added to the water which maintained the humidity. The hypocotyl segments (0.7-1.0 cm) under the apical hook were used to isolate the protoplasts.

Isolation and purification of protoplasts

The method of Roy and De (1983) was modified to isolate the protoplasts. The hypocotyl segments were cut twice longitudinally, and treated with CPW 13M (pH 5.8) solution for 1 h. These excised sections were incubated in CPW13M (pH 5.8) containing 2.0 % cellulase (Onozuka RS), 0.3% hemicellulase, and 0.5% Macerozyme (Onozuka R-10) for 14 h at 27± 1 ℃ in complete darkness. After incubation, the crude protoplast suspension was filtered through 300 μm stainless mesh to discard the cell debris. Protoplasts were washed twice with CPW13M (pH 5.8) solution and purified further by sucrose density gradient with CPW21S (pH 5.8) solution. The number of purified protoplasts was counted with the hemocytometer.

Measurement of ACC-induced ethylene production

The isolated protoplasts (10^6) were incubated in 2 mL Mes-Tris buffer (2×10^{-2}, pH 5.8) in a 10 mL vial containing 3×10^{-4} M ACC, 0.4 M sucrose, and 5×10^{-5} g/mL chloramphenicol for 6 h at 27± 1℃ in complete darkness. The concentration of Ca^{2+} in the medium was 5×10^{-3} M as the same to CPW solution. No more Ca^{2+} was further added. A 1 mL sample was taken to measure the ethylene production using a gas chromatography (Shimazu GC-R1 A, 3 m stainless column; air, 0.4 kg/cm^2; carrier (N_2), 50 mL/min; H_2, 0.6 kg/cm^2) equipped with the active alumina column (80/100 mesh). Represented data mean the percentage which were measured on the basis of control in the ethylene production.

Protoplast vitality

The protoplast suspension (100 μL) was mixed with the equal volume of the 0.5% Evans blue solution. Ten μL of these mixture was applied to determine the vitality. The ratio of protoplast number in the hemocytometer (unstained number/total number) is calculated for the vitality.

RESULTS

Characteristics of protoplasts

The ACC-induced ethylene production from the

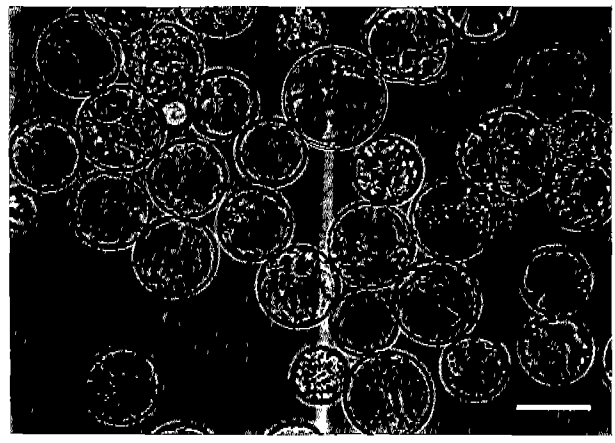

Fig. 1. Purified protoplasts from 2.5 day old mungbean hypocotyls. Hypocotyl segments were pretreated with CPW13M solution for 1 h. CPW13M solution was replaced by mixture of 2.0% cellulase, 0.3% hemicellulase and 0.5% macerozyme. Protoplasts were washed twice with CPW13M solution after incubation for 14 h in the enzymetic mixture. The process of purification was carried out with CPW21S solution. Bar=40 μm.

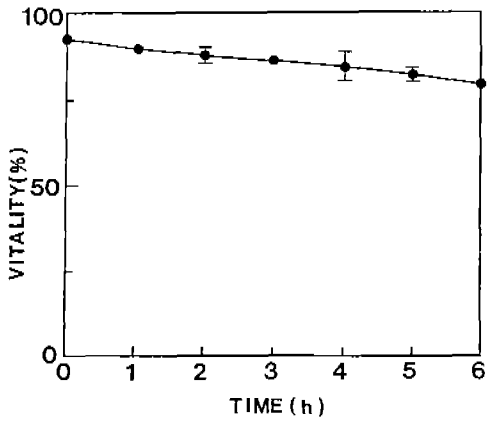

Fig. 2. The vitality of purified protoplasts. The protoplast-suspension (100 μL) was mixed with the same volume of 0.5% Evans blue solution. This mixed solution was used to determine the vitality of protoplasts.

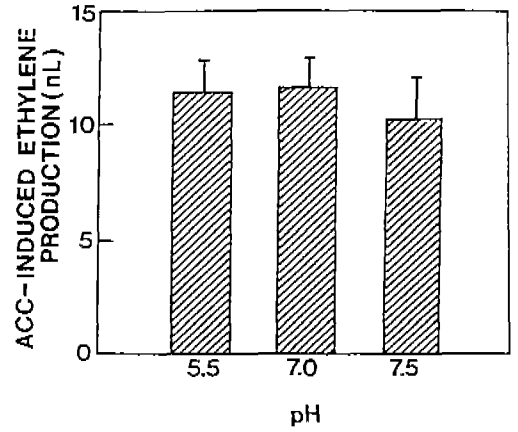

Fig. 3. Effect of pH on the ACC-induced ethylene production in 10^6 protoplasts which were incubated for 6 h in the presence of 3×10^{-4}M ACC.

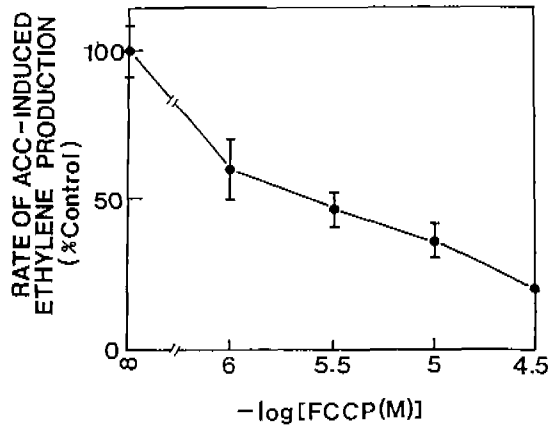

Fig. 4. Effect of FCCP on the ACC-induced ethylene production in 10^6 protoplasts which were incubated for 6 h in the presence of 3×10^{-4}M ACC.

hypocotyls which were grown on the agar with 10^{-2} M $CaCl_2$ was 40-45% higher than that of hypocotyls which were grown on the agar without Ca^{2+}. The latter tissues were sensitive to the plasmolysis and ACC-induced ethylene production was inhibited by 70% through the plsmolysis (Seo and Oh, 1993). Ca^{2+} has been known to enhance the membrane stability (Apelbaum *et al.*, 1981). The hypocotyls grown on the agar containing 10^{-2}M $CaCl_2$ was used in this experiment beacuse Ca^{2+} maintains the membrane integrity during isolation and enhances the ethylene production in protoplasts.

The number of the purified protoplasts (Fig. 1) was 2.1×10^6/g (fr wt) with 92% vitality. The vitality of protoplasts was slowly reduced to 80% after 6 h incubation as shown in Fig. 2.

The effect of pH in the incubation medium was examined on the ACC-induced ethylene production in protoplasts (Fig. 3). There was no difference between pH 5.5 and pH 7.0. However, the ethylene production showed 10% reduction at pH 7.5 when compared to the ethylene production at pH 7.0. From these results, we determined the pH of incubation medium as 5.8 which was the same to the CPW13M soultion. The ethylene production from the protoplasts (10^6) was 13.4± 0.8 nL in the presence of 3×10^{-4}M ACC for 6 h.

Effect of H^+-gradient established across plasma membrane on ACC-uptake

When the exogenous ACC is moved into the cytosol through H^+/amino acid-symporter in the plasma membrane (Bush and Langston-Unkefer, 1988), H^+-gradient should be established across the plasma membrane. The possibility that the ACC-uptake into the protoplasts was dependent on the H^+-gradient established across the plasma membrane was examined in the presence of FCCP (carbonycyanide-*p*-trifluromethoxyphenylhydrazone), a proton translocator, and *o*-vanadate, a specific inhibitor of plasma membrane H^+-ATPase.

The treatment with 10^{-6}M and 3×10^{-5}M FCCP inhibited the ACC-induced ethylene production to 60% and 20% of the control (Fig. 4). And the application of 10^{-5}M and 10^{-4}M vanadate inhibited the ACC-induced ethylene production to 85% and 40% of control, respectively (Fig. 5).

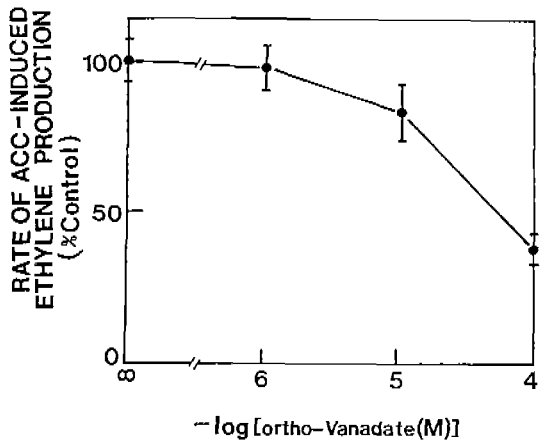

Fig. 5. Effect of *o*-vanadate on the ACC-induced ethylene production in 10^6 protoplasts which were incubated for 6 h in the presence of 3×10^{-4}M ACC.

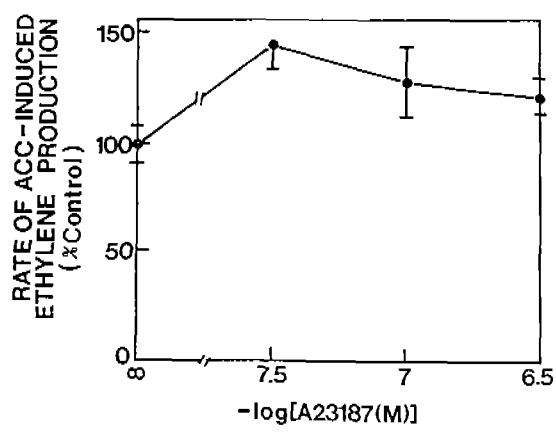

Fig. 7. Effect of A23187 on the ACC-induced ethylene production in 10^6 protoplasts which were incubated for 6 h in the presence of 3×10^{-4}M ACC.

Effect of Ca^{2+} on ACC-induced ethylene production

The possibility that Ca^{2+} moves into the cytosol through Ca^{2+}-channel with the ACC-uptake and consequently stimulates ACC oxidase activity in the cytosol was examined. The ACC-induced ethylene production was measured when Ca^{2+} influx is affected by the treatment with verapamil and A23187.

The cytosolic Ca^{2+} concentration could be reduced by the treatment with verapamil which inhibits Ca^{2+} influx by binding to Ca^{2+}-channel (Andrejauskas *et al.*, 1985). The ACC-induced ethylene production in protoplasts was reduced by 10 to 30% when 10^{-5}M and 10^{-4}M verapamil was applied. Moreover, the treatment with 10^{-3}M verapamil inhibited the ethylene production by over 65% (Fig. 6).

Since 5×10^{-3}M $CaCl_2$ was present in the incubation medium, we could increase the Ca^{2+} concentration of protoplasts cytosol artificially by the treatment with Ca^{2+}-ionophore, A23187. The treatment with 3×10^{-8}M and 3×10^{-7}M A23187 stimulated the ethylene production to 145% and 125% of control, respectively (Fig. 7).

EGTA [ethylene glycol-bis(β-aminoethyl ether) *N, N,N',N'*-tetraacetic acid], a Ca^{2+}-chelator, reduces the Ca^{2+} influx into the cytosol in protoplasts. Therefore, the concentration of cytosolic Ca^{2+} could be reduced by the treatment with EGTA. The application of 10^{-5}M to 10^{-3}M EGTA inhibited the ACC-induced

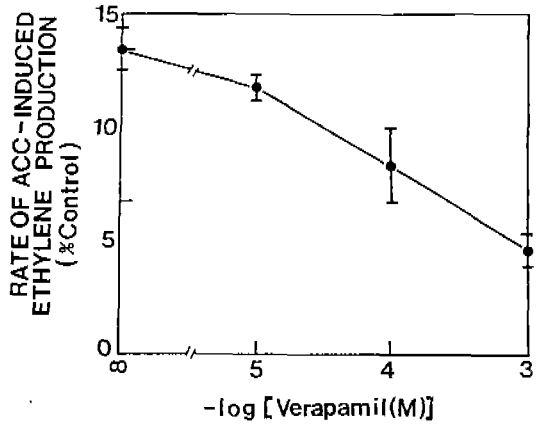

Fig. 6. Effect of verapamil on the ACC-induced ethylene production in 10^6 protoplasts which were incubated for 6 h in the presence of 3×10^{-4}M ACC.

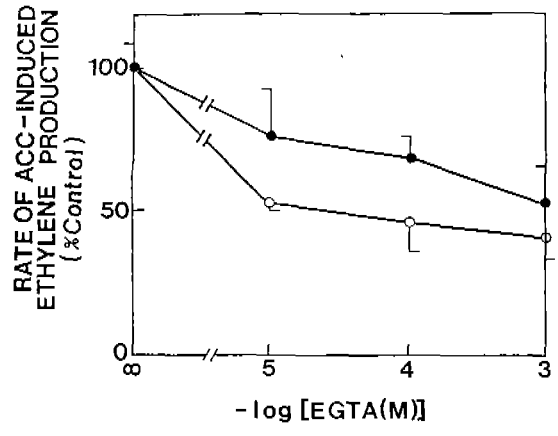

Fig. 8. Effect of EGTA on the ACC-induced ethylene production in 10^6 protoplasts which were incubated with 3×10^{-7}M A23187 (open circle) and without A23187 (closed circle) in the presence of 3×10^{-4}M ACC.

ethylene production by 30 to 40%. This inhibition effect of EGTA in the ethylene production magnified in the presence of A23187 (Fig. 8).

DISCUSSION

When the protoplasts (10^6 cells) were incubated in the incubation medium for 6 h, the pH changed from pH 7.0 and 7.5 to 6.3 and 6.5, respectively (data not shown). This pH change is an evidence that the primary transport system maintains the activity in the plasma membrane during incubation. In contrast, the pH in the incubation medium was changed from 5.5 to 5.8 during the incubation, suggesting that the homeostasis of the H^+-gradient established in the plasma membrane would maintain.

The treatment with 3×10^{-5} M FCCP, the proton translocator, reduced the ACC-induced ethylene production by 80% in protoplasts (Fig. 4). This result is consistent with the inhibition of the ACC-induced ethylene production by DNP in protoplasts isolated from sunflower seedlings (Bailly *et al.*, 1993). Depolarized membrane potential due to H^+ influx through the ACC/H^+-cotransporter should be repolarized for the continuous ACC-uptake. Therefore, inhibition of the activity of plasma membrane H^+-ATPase known to maintain the membrane potential (Leonard, 1988) could reduce the ethylene production. The treatment with 10^{-4} M *o*-vanadate, a specific inhibitor of plasma membrane H^+-ATPase, reduced the ACC-induced ethylene production by 60% (Fig. 5). The same concentration of *o*-vanadate inhibited the activity of partially purified plasma membrane H^+-ATPase by 90% from the tomato root (Anthon and Spanswick, 1986). This discrepancy in the rate of inhibition could be resulted from either the different status of H^+-ATPase in each experiment or the participation of the other primary transport system such as redox-system to maintain the membrane potential. These results (Figs. 4 and 5) suggested that the exogenous ACC-uptake into protoplasts could be, at least in part, dependent on the H^+-gradient established in the plasma membrane.

Since loosly-bound cell wall proteins would be removed during the protoplasts preparation (Christoffersen *et al.*, 1993), the influence of ACC oxidase located in the apoplasm (Latché *et al.*, 1993) in the ethylene production was ruled out in this study. And the ACC-induced ethylene production in protoplasts was enhanced when the amount of transported Ca^{2+} into protoplasts from the medium increased. These data suggested that ACC-induced ethylene production in protoplsts was affected by ACC oxidase located in the cytosol.

The ACC-induced ethylene production in protoplasts isolated from sunflow hypocotyls was inhibited by calmodulin antagonists (Bailly *et al.*, 1993). This result suggested that transported Ca^{2+} into the cytosol through the Ca^{2+}-channel could bind to calmodulin and consequently stimulate the ACC oxidase activity.

The results presented in this study are consistent with the hypothesis that ACC oxidase is located in the cytosol. Additionally, these data demonstrate that Ca^{2+} influx in the plasma membrane are essential for the stimulation of ACC oxidase activity.

ACKNOWLEGEMENTS

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綠豆 原形質體에서 H^+ 濃度勾配에 依存하여 吸收된 1-Aminocyclopropane-1-Carboxylic Acid의 에틸렌 轉換에 미치는 Ca^{2+}의 效果

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적 요

2.5일된 녹두 하배축으로부터 획득한 원형질체를 사용하여, 첨가된 1-aminocyclopropane-1-carboxylic acid (ACC)의 흡수가 원형질막을 경계로 형성되어 있는 H^+ 농도구배에 의존할 가능성을 검토하였다. Carbonycyamide-*p*-trifluromethoxyphenylhydrazone (FCCP)를 사용하여 H^+ 농도구배를 붕괴시킬 경우 ACC에 의한 에틸렌 생성은 억제되었다. 또한, 원형질막 H^+-ATPase의 억제제인 *o*-vanadate에 의해서도 에틸렌 생성은 억제되었다. Ca^{2+}-channel blocker인 verapamil과 Ca^{2+}-chelator인 ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA)를 사용하여 원형질체 내로의 Ca^{2+} 이동을 감소시킬 경우 ACC에 의한 에틸렌 생성은 억제되었다. 반면에, Ca^{2+}-ionophore인 A23187를 사용하여 원형질체 내로의 Ca^{2+} 이동을 증가시킬 경우 에틸렌 생성은 촉진되었다. EGTA에 의한 에틸렌 생성 억제 효과는 EGTA와 A23187를 동시에 처리할 경우 더욱 뚜렸해졌다. 이상의 사실로, *in vitro*에서 ACC가 원형질막을 경계로 형성된 H^+ 농도구배를 사용하여 흡수될 때, 동시에 원형질체 내로 이동한 원형질체 밖의 Ca^{2+}에 의하여 cytosol에 위치한 ACC oxidase의 활성이 촉진될 가능성을 생각할 수 있다.

주요어: ACC 흡수, H^- 농도구배, Ca^{2+}, ACC에 의한 에틸렌 생성, ACC oxidase

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