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Suppression of Ca^{2+}-Influx after ACC-Uptake by Spermine in *Vigna radiata*

Lee, Sang-Choon, Min Seon Choi, Sun Hi Lee[1] and Seung-Eun Oh*

Department of Biology, Kon-Kuk University, Seoul 143-701, Korea
[1]Department of Biology, Yonsei University, Seoul 120-749, Korea

Exogenous ACC-induced ethylene production in mung bean hypocotyl segments was severely inhibited by the treatment of spermine in a 6 hr incubation. In protoplasts acquired from the same tissues and pretreated with ACC, the concentration of cytosolic Ca^{2+} was increased after the addition of exogenous Ca^{2+}. However, this increase of Ca^{2+} concentration was strongly suppressed by spermine. In previous studies, an artifical increase of Ca^{2+} influx by the treatment of a Ca^{2+}-ionophore stimulated ACC-induced ethylene production. The inhibitory effect of spermine on ACC-induced ethylene production was more prominent than that of putrescine which has fewer NH^{3+} groups than spermine. In addition, spermine more prominently suppressed ACC-induced ethylene production in protoplasts in which fewer Ca^{2+} ions were released from Ca^{2+}-storage organelles. Also, the amount of transcript of ACC-oxidase which converts ACC to ethylene was decreased by the treatment of spermine. However, this reduction resulted only through the suppression of ethylene production in a 2 hr incubation of mung bean segments. On the basis of these results, we suggest that there is a coupling of ACC-uptake to the increase of cytosolic Ca^{2+} concentration. In addition, the reduction of exogenous ACC-induced ethylene production by spermine could have resulted, at least partially, from reducing the Ca^{2+} influx which stimulates ACC-oxidase activity.

Keywords: ACC, ACC-oxidase, ethylene, Ca^{2+}, spermine

An immediate precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) is transported from synthesizing sites to perception organs through the vascular system and triggers physiological responses such as epinastic curvature of the leaves after conversion to ethylene (Bradford and Yang, 1980, Voesenek *et al.*, 1990). Although investigation of the interorgan transport of ACC is essential for understanding the roles of ethylene, there are few data compared to the transport of other phytohormones with the exception of those involving root-to-shoot translocation (Finlayson *et al.*, 1991). It is also suprising that the mechanisms of the uptake and conversion of ACC to ethylene in the perception cells are not clearly addressed. In some tissues, conversion of exogenous ACC to ethylene that mimicked the uptake and conversion of transported ACC in perception cells was stimulated by the treatment of exogenous Ca^{2+} (Burns and Evensen, 1986). However, exogenous polyamines that are polycations at physiological pH levels inhibited this conversion (Fuhrer *et al.*, 1982; Evans and Malmberg, 1989). Variation in the stability of the membrane structure and the maintenance of membrane integrity following the treatment of such chemicals were discussed as the explanations for such effects. These explanations pivoted on the assumption that ethylene production from ACC is a membrane associated step (Guy and Kende, 1984). However, recently, such interpretations have been challenged by purification of ACC-oxidase *in vitro* (Smith *et al.*, 1992), and by analysis of the DNA sequence encoding ACC-oxidase (Dong *et al.*, 1992). On the basis of these results, ACC-oxidase could be localized in cytosol instead of in the membrane (Christoffersen *et al.*, 1993). Therefore, the regulatory effects of Ca^{2+} and polyamines on the conversion of exogenous ACC to ethylene shoud be re-examined in other respects. In previous works using mung bean hypocotyl tissues and protoplasts, an artificial increase of cytosolic Ca^{2+} promoted ACC-induced ethylene production. In contrast, the treatment of a calcium-channel

*Corresponding author: Fax +82-2-3436-5432

blocker strongly suppressed ethylene production (Seo *et al.*, 1994). Therefore, we suggest that the activity of ACC-oxidase which converts ACC to ethylene could be stimulated by an increase of cytosolic Ca^{2+}. In this work, we present the possible coupling of exogenous ACC-uptake with the increase of cytosolic Ca^{2+} which stimulates the ACC-oxidase localized in cytosol. Also, the inhibitory effect of a polyamine, specifically spermine, on exogenous ACC dependent ethylene production will be discussed in respect to reducing the cytosolic Ca^{2+} increase.

MATERIALS AND METHODS

Plant material

Mung bean (*Vigna radiata* W. cv. Sunhwa) seeds were soaked overnight in tap water and germinated on agar plates with or without 10 mM $CaCl_2$ for 2.5 days at 27°C in darkness. The humidity was kept at over 90%. For the elimination of ethylene effects on the seedling's growth, 4×10^{-5} M $KMnO_4$ was added to the water that maintained the humidity. The hypocotyl segments under the apical hook were used. GAC- and GA- tissues mean segments acquired from seedlings grown on agar with and without $CaCl_2$, respectively.

Isolation of protoplasts

The method of Seo *et al.* (1994) was used to isolate the protoplasts from GAC-tissues of mung bean. Isolated protoplasts were further separated by Ficoll step gradient according to the method of Masuda *et al.* (1989). After centrifugation, the vacuole rich VR-protoplasts were acquired from between the suspension solution and the 4% Ficoll/9M solution. The cytoplasm rich CR-protoplasts were acquired between the 4% and 8% Ficoll/9M solution. The viability of the protoplasts was tested by staining with 0.5% Evans blue solution.

Determination of ethylene production

GA-tissues (0.2 g) were incubated in 1 ml Mes-Tris buffer (10 mM, pH 7.2) containing 0.3 mM ACC in a 10 mL vial. The isolated protoplasts (5×10^5) were incubated in 0.5 mL Mes/Tris buffer (20 mM, pH 5.8) containing 0.5 mM ACC and 1 mM $CaCl_2$ in a 5 mL vial. After a 6 hr incubation, a 1 mL sample was taken to measure the ethylene production using a gas chromatograph (Shimazu GC-R1) equipped with an active alumina column (80/100 mesh).

Detection of the fluorescence signal in Fura-2 AM loaded protoplasts

The method of Chae *et al.* (1990) was modified to detect the fluorescence signals in protoplasts after treatment with chemicals. Isolated protoplasts were washed twice with CPW 9M/Mes solution without $CaCl_2$ and resuspended with HEPES buffer (20 mM, pH 5.8) including 1 mM KCl, 1 mM MgSO4, 0.05% BSA, 9% mannitol, and 50 μM Fura-2 AM. After loading of the dye for 1 hr at 27°C, the protoplasts were washed with HEPES buffer without Fura-2 AM. Fluorescence signals were detected by fluorescence spectrophotometer (Hitachi F-2000) and analyzed by software from Hitachi. The wavelengths of extinction and emission were 340 nm and 510 nm, respectively.

Northern blot analysis of ACC-oxidase transcript

Total RNA in mung bean hypocotyl segments was isolated by an RNeasy Plant Kit (Qiagen) according to the manufactures manual. Total RNA (30 μg each) was separated on a formaldehyde gel and blotted onto a Hybond-N+ membrane (Amersham). The 0.9 kb ACC-oxidase cDNA isolated from mung bean (Kim and Yang, 1994) was labeled with Gene Images Kit (Amersham) and hybridized with RNA on a membrane. Signals were detectable after a 1 hr exposure of the blot to Hyperfilm-ECL (Amersham).

RESULTS

Inhibition of ACC-induced ethylene production by polyamines

The effect of polyamines on exogenous ACC-induced ethylene production in mung bean hypocotyls was examined (Fig. 1). Both polyamines tested in this study inhibited ethylene production at a range of 0.1-1 mM. Spermine, which has four NH^{3+} groups, inhibited ACC-induced ethylene production more severely than putrescine which has only two NH^{3+} groups. While the treatment of putrescine resulted in a 50-80% reduction of ethylene production, spermine reduced it 60-95% in the GA-tissues. These inhibitory effects of polyamines detected in GA-tissues were diminished in GAC-tissues which were grown on agar containing 10 mM $CaCl_2$. In previous studies, the inhibition rate of ACC-induced ethylene production in GA-tissues resulting from the treatment of a calcium-chelator, EGTA, and calcium-channel blockers such as $LaCl_3$ and verapamil, was reduced in GAC-tissues (Seo and Oh, 1993). In the same GAC-tissues, spermine reduced ethylene production 50-90%

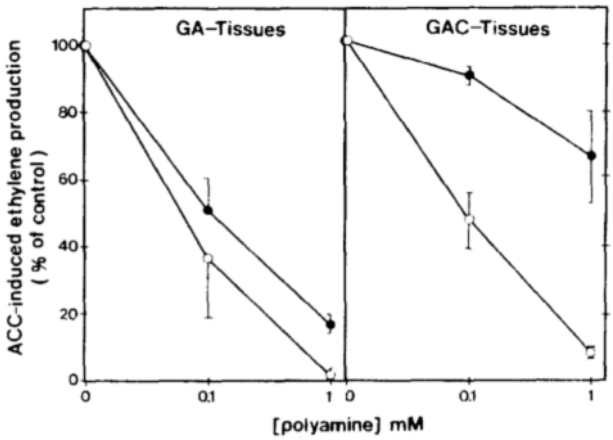

Fig. 1. Effects of putresine (closed circle) and spermine (open circle) on exogenous ACC-induced ethylene production in GAC- and GA-tissues in the presence of 0.3 mM ACC. GAC-tissues were acquired from seedlings grown on agar with 10 mM $CaCl_2$. GA-tissues were acquired from seedlings grown on agar without $CaCl_2$. Incubation was carried out at 27°C for 6 hr. Vertical bars indicated standard errors.

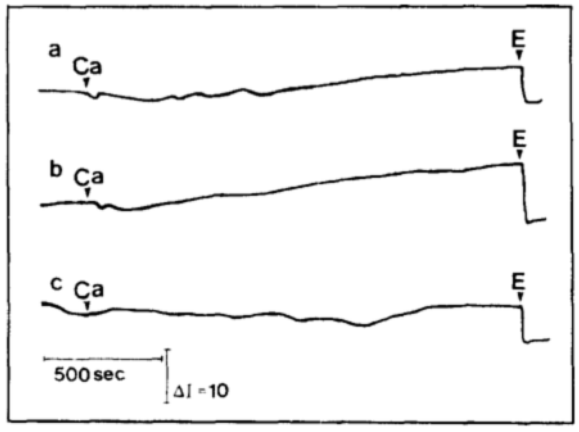

Fig. 2. The variation of the cytosol Ca^{2+} concentration was determined by fluorescence spectrophotometer. a, without pretreatment with ACC or the addition of spermine; b, pretreatment with 0.5 mM ACC for 20 min; c, treatment with 1 mM spermine after the pretreatment with ACC. Ca means addition of 3 mM $CaCl_2$. E means addition of 6 mM EGTA.

and putrescine reduced it 10-35%.

Blocking the increase in cytosolic Ca^{2+} by spermine

As described above, in mung bean hypocotyls, manipulating the influx of Ca^{2+} by treatment of chemicals such as a calcium-ionophore, -chelator, or -channel blocker stimulated or inhibited ACC-induced ethylene production. Accordingly, we tested whether or not the inhibitory effects of polyamines were the results of blocking the increase of cytosolic Ca^{2+}. For measuring the variation of cytosolic Ca^{2+} concentration, we used a calcium-specific fluorescent dye, Fura-2 AM. This dye easily permeates the membrane and converts to impermeable Fura-2 by the activity of esterase in the cytosol (Haugland, 1996). The relative intensities of fluorescence resulting from the complexes of calcium ions and fluorescent dyes localized in the cytosol did not increase immediately. After the addition of 3 mM $CaCl_2$ (Fig. 2a), there was a 10 min lag period before fluorescence intensities increased. In contrast, the relative fluorescence intensity from protoplasts pretreated by 0.5 mM ACC increased immediately after the addition of $CaCl_2$ (Fig. 2b). This steady increase of fluorescence intensity was diminished by the treatment with 1 mM spermine (Fig. 2c). In this case, only a slight increase of fluorescence intensity was detected after 20 min. The promotion of cytosolic Ca^{2+} was also detected in the process of ABA-induced stomata closing (Ward *et al.*, 1995). Although it has been suggested that a Ca^{2+}-influx from the extracellular space is required for stomata closing, some works indicate that Ca^{2+} is released in parallel from intracellular stores by depolarization of the plasma membrane (Gilroy *et al.*, 1990). The release of Ca^{2+} from storage organelles, especially vacuoles was confirmed by Ca^{2+} imaging studies (McAinsh *et al.*, 1992). In this context, we examined whether the Ca^{2+} released from storage organelles after an ACC-influx could participate in the promotion of exogenous ACC-induced ethylene production. For the purpose of this experiment, we further separated isolated protoplasts from mung bean hypocotyls into two types depending on the presence, or abscence of a central vacuole. The separated vacuole rich (VR)-protoplasts were heterogeneous in size (Fig. 3c, ranging from 35 to 60 μm) and most of the VR-protoplasts had well-developed large vacuoles according to observation by phase-contrast microscope (Fig. 3e). However, cytoplasm rich (CR)-protoplasts were more homogeneous in size and smaller (Fig. 3d, 20-25 μm) than VR-protoplasts and central vacuoles were not observed in most CR-protoplasts (Fig. 3f). In VR-protoplasts, the stimulatory effect of tapsigagin, a specific inhibitor of endoplasmic reticulum (ER) Ca^{2+}-ATPase (Thastrup *et al.*, 1990) was not seen (Table 1). This chemical is used for the artificial increase of cytosolic Ca^{2+} in animal cells. In contrast, about a 10% increase in ethylene production was seen in CR-protoplasts by the treat-

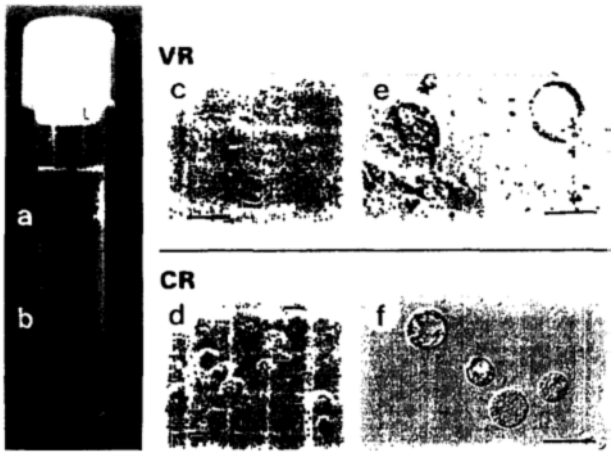

Fig. 3. The photographs of isolated protoplasts without (c and e, the bar represents 50 μm) and with phase-contrast optics (d and f, the bar represents 30 μm). VR means vacuole rich protoplasts acquired from band (a) between the suspention solution and the 4% Ficoll/9M solution after centrifugation for 10 min at 150 g. CR means cytoplasm rich protoplasts acquired from band (b) between the 4% and Ficoll 9M solution.

Table 1. The effect of tapsigargin (Tap), 3,4,5,-trimethoxybenzoic acid 8-(diethylamino)-octyl ester (TMB-8) and spermine (Spm) on the exogenous ACC-induced ethylene production in two different types of mung bean hypocotyl protoplasts.

Treatments	Ethylene production (%) in	
	Vacuole rich protoplasts	Cytoplasm rich protoplasts
Control	100	100
Tap (10^{-5} M)	100±3	109±1
TMB-8 (10^{-4} M)	94±3	87±2
Spm (10^{-4} M)	57±5	71±5

ment of tapsigagin. The production of ethylene in VR-protoplasts was affected slightly by the treatment of TMB-8 which is known to inhibit Ca^{2+} release from storage organelles (Salzer *et al.*, 1996). However, the ethylene production in CR-protoplasts was reduced by more than 10%. The inhibition rate by treatment of 1 mM spermine in CR-protoplasts was about 30%. However, the inhibitory effect of spermine in VR-protoplasts which were less sensitive to the treatment of TMB-8 and tapsigagin, increased by over 40% (Table 1).

Reduction of the amount of the ACC-oxidase transcipt amount by spermine

We tested whether spermine could also affect *de novo* synthesis of ACC-oxidase in mung bean hypocotyl during a 6 hr incubation, especially, in the process of transcription. As shown in Fig. 4, the level of ACC-oxidase transcript was strongly suppressed by spermine. However, in the prescence of an ethylene action inhibitor, 2,5-norbornadiene (NBD), the detected amount of transcript of ACC-oxidase gene prominently decreased. Interestingly, the inhibitory effect on the transcription level of spermine was not detected in the presence of NBD. On the basis of these results, decreases in amounts of ACC-oxidase gene transcript could only be a result of the suppression of ethylene production by spermine. We tried to determine when the decrease of transcription

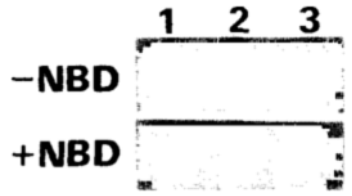

Fig. 4. Northern blot analysis of ACC-oxidase transcript in mung bean hypocotyl segments in the presence or absence of 1000 ppm 2,5-norbornadiene (NBD). 1, control; 2, treatment with 0.3 mM ACC; 3, treatment with 0.3 mM ACC and 1 mM spermine.

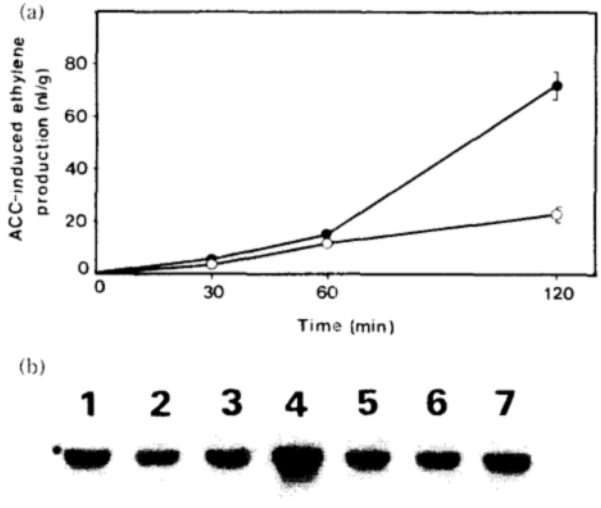

Fig. 5. Time course of the exogenous ACC-induced ethylene production after treatment with (open circle) and without (closed circle) 1 mM spermine (a) and the amount of ACC-oxidase transcript (b) in mung bean hypocotyo segments in the presence of 0.3 mM spermine. Incubation was carreid out at 27°C. Vertical bars indicate standard errors. 1, control; 2, at 30 min; 3, at 60 min; 4, at 120 min; 5, at 30 min after treatment of 1 mM spermine; 6, at 60 min after treatment of 1 mM spermine; 7, at 120 min after treatment of spermine.

of ACC-oxidase gene by the reduction of ethylene production was detectable during the incubation period of mung bean segments. The amount of ethylene production was slightly suppressed by the treatment of spermine in even a short period of incubation (Fig. 5a). In a 1 hr incubation, the difference in the amount of ACC-oxidase gene transcript was not detectable in segments whether treated with spermine or not (Fig. 5b). However, the difference in the level of transcript detected increased accordingly, after prominent suppression of ethylene production in segments treated with spermine after further incubation of 1 hr.

DISCUSSION

The mechanism of the influx of ACC into the cytosol through the plasma membrane has not been thoroughly studied. However, there are some data explaining the intracellular transport of ACC through the tonoplast into the vacuole (Saftner and Mehta, 1990; Saftner and Martin, 1993). According to these data, the uptake of ACC into isolated maize mesophyll vacuoles was carrier mediated and dependent upon an electrochemical potential gradient for protons (Saftner and Martin, 1993). On the basis of these mechanisms of intracellular transport of ACC, the following assumption has been adapted to this work. Exogenous ACC could be taken into the cytosol through the H^+/amino acid-symporter on the plasma membrane along with other neutral amino acids (Bush, 1993), and the membrane potential could be depolarized by the influx of H^+ after ACC uptake into the cytosol. The change of membrane potential is known to open the voltage-operated Ca^{2+}-channels in the plasma membrane and release the stored Ca^{2+} (Ward *et al.*, 1995). Therefore, the increase of the cytosolic Ca^{2+} concentration resulting from the above assumed sequence of events was proved after the uptake of endogenous ACC by determining the cytosolic Ca^{2+} change (Figs. 2a and 2c). The detection method applied in this work using a Fura-2AM was sensitive enough to detect the relative variation of cytosolic Ca^{2+} in mung bean protoplasts. According to the analysis of the fluorescence intensity by the treatment of high Ca^{2+} and Triton X-100, the membrane permeability of the protoplasts used in this work seemed to be well conserved during the experiment (data not shown). While the rapid and dynamic change of the cytosolic Ca^{2+} was elicited by cold shock in tobacco and *Arabidopsis* protoplasts (Knight *et al.*, 1996), the detected change of cytosolic Ca^{2+} after ACC-uptake in this work was not so dynamic, but persisted longer. This tendency of variation of cytosolic Ca^{2+} was also detected in *Commelina* guard cells after treatment of high concentrations of exogenous Ca^{2+} (Gilroy *et al.*, 1991). The responses of two types of protoplasts that were in different developmental states in respect to the presence of a central vacuole, differed against the artificial stimulation or inhibition of released Ca^{2+} from Ca^{2+}-storage organelles (Table 1). While central vacuoles are known to be a main Ca^{2+}-storage organelle in most plants cells (McAinsh *et al.*, 1992; Canut *et al.*, 1994), the inhibitory effect of artificially blocking Ca^{2+} release upon the conversion of exogenous ACC was unexpectedly more prominent in CR-protoplasts than in VR-protoplasts which have well developed central vacuoles. In addition, the effect of tapsigagin was not detected in VR-protoplasts. Therefore, the stored Ca^{2+} could be released from other storage organelles such as the endoplasmic reticulum (ER) after ACC uptake, at least in CR-protoplasts, and could be involved in the promotion of ethylene production in CR-protoplasts. However, the effect of increasing or decreasing Ca^{2+} release on ethylene production was about 10%. The inhibitory effect of spermine on exogenous ACC-induced ethylene production was more prominent in VR-protoplasts (Table 1). In this case, the difference in the inhibtion rate was also about 10%. So, we suggest that the inhibitory effect of spermine on ethylene production could be the result of blocking the Ca^{2+} influx to the plasma membrane. Of course, we could not rule out the possibility that the reduction of the amount of ACC taken up into the cytosol was due to the treatment of spermine. According to our assumption, this reduction of ACC-uptake could also decrease the Ca^{2+}-influx and/or Ca^{2+}-release from storage organelles. As a result of this coupling event, ACC-induced ethylene production could be severely suppressed. However, in this work, we mainly addressed whether the cytosolic Ca^{2+} concentraion could be influenced by the treatment of spermine. The inhibitory effects of the two polyamines examined were dependent on the number of NH^{3+} groups in each chemical structure. These highly protonated polycations interact with anionic charged membrane phospholipids. In zucchini hypocotyl, the binding of spermine to the plasma membrane is known to be quite specific (Tassoni *et al.*, 1996). However, as of now, we do not know whether the binding of spermine on the plasma membrane of mung bean hypocotyl cells is specific and is the exact blocking mechanism of Ca^{2+} influx after

binding to the plasma membrane. The inhibitory effects of polyamines used in this experiment were diminished in GAC-tissues (Fig. 1). On the basis of this result, we suggest that the amount of influx of Ca^{2+} from apoplasts in GAC-tissues could be higher than that in GA-tissues. In this context, the reduced effect of spermine in GAC-tissues could be interpreted in respect to the increase in cytosolic Ca^{2+} concentration from apoplasts and/or Ca^{2+} storage organelles.

We present in this work the possibility of coupled ACC-uptake and increased cytosolic Ca^{2+}. The physiological meaning of this coupling is that the transported ACC could be effectively converted to ethylene in perception organs. The detected increase of cytosolic Ca^{2+} could be the result (Fig. 2) mainly of influx from apoplasts after ACC uptake. Also, cytosolic Ca^{2+} could increase partially by the release of stored Ca^{2+} (Table 1). This increse of Ca^{2+} could stimulate ACC-oxidase which converts ACC to ethylene in most plant tissues. In protoplasts isolated from sunflower hypocotyls, ACC-induced ethylene production was inhibited by the treatment of calmodulin antagonist (Bailly *et al.*, 1993). This result suggestses that increased cytosolic Ca^{2+} after uptake of ACC could bind to calmodulin and consequently stimulate ACC-oxidase. Then, the promoted ethylene production driven by the increase of ACC and the activation of ACC-oxidase could promote transcription of ACC-oxidase between 60 min and 120 min (Fig. 5) in an autostimulatory manner as in carnation flower (Woodson *et al.*, 1993). This would further stimulate the conversion of uptaken ACC to ethylene.

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