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Action of Calcium on Ethylene Biosynthesis Induced by Auxin and Cytokinin in Mungbean Hypocotyl Segments

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녹두하배축에서 Auxin과 Cytokinin에 의한 에틸렌 생합성에 대한 Ca^{2+}의 작용

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ABSTRACT

Calcium promoted ethylene production from mungbean hypocotyl segments incubated in the presence of either auxin or cytokinin (kinetin). Time course studies indicated that the calcium effect on ethylene production had a longer latent period (about 6 h) in combination with kinetin than with auxin. Studies on the effects of agents that are known to interfere with either action or transport (uptake) of calcium on ethylene biosynthesis indicated different patterns between auxin- and kinetin-treated tissues. Auxin-induced ethylene production was inhibited by the calmodulin inhibitor, trifluoperazine (TFP), and this inhibition was overcome by high concentrations of calcium applied, but TFP had no significant effect on kinetin-induced ethylene production regardless of calcium in the medium. The calcium channel blocker, verapamil, inhibited auxin-induced, but had little effect on kinetin-induced, ethylene production. In vivo activity of "ethylene forming enzyme (EFE)" was found to be substantially promoted by calcium treatment. The enzyme activity was further increased by kinetin when segments were simultaneously treated with calcium, but auxin did not have such an effect.

INTRODUCTION

Calcium plays an important role in the control of cellular processes. In plants, calcium is known to mediate a variety of metabolic and developmental processes, especially in relation to hormonal actions (cf. Hepler and Wayne, 1985). Direct and indirect evidence from the studies employing specific inhibitors indicate involvement of calcium in such auxin-mediated responses as cell elongation (Elliot, 1983; Raghothama, *et al.*, 1985), gravitropism (Lee *et al.*, 1983, a,b) and leaf abscission (Poovaiah, *et al.*, 1988). Calcium is also involved in cytokinin-regulated

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developmental proccsses such as bud formation in *Funaria*(Saunderrs and Hepler, 1983), leaf senescence (Poovaiah, 1987), and growth of cultured callus cells (Elliott, 1983). Molecular and/or biochemical approaches to problcms of calcium action in plants are also well documented, e.g. on protein phosphorylation (Morré *et al.*, 1984; Poovaiah *et al.*, 1987) and membaranc organizatioon (Paliyath *et al.*, 1984).

Investigators of calcium action on ethylene biosynthesis in plant tissues reported contradictory results. Calcium was reportcd, on one hand, to promote ethylene production in a variety of tissues (Burns and Evenson, 1986; Hasenstein and Evans, 1986; Cheverry *et al.*, 1988; Sanchcz-Calle *et al.*, 1989), but on the other hand, calcium treatmcnt was reported to have resulted in an inhibition of cthyylene biosynthcsis (Lieberman and Wang, 1982; Ruth *et al.*, 1982) in other cases.

The biosynthetic pathway of cthylene in higher plants has been well established (Adams and Yang, 1979), and the action of auxin to stimulate ethylene biosynthesis has been well elucidated on the basis of auxin-induction of a key enzyme in the biosynthetic pathway (Yu *et al.*, 1979; Yoshii *et al.*, 1980). Cytokinin also promotcs ethylene production, but the cytokinin effect is known as being syncrgistic in the presence of auxin (Imasecki *et al.*, 1975) and its underlying mechanism is not known at the present (Yoshii and Imaseki, 1981).

In this repoort, we present rcsults of our studics on involvement of calcium in ethylene production induced by cither auxin or kinetin in an efffort to characterize the nature of interactions betwcen calcium and the hormones.

MATERIALS AND METHODS

Plant material. One cm segments were excised from the subapical hypoocotyl of etiolated mungbean (*Vigna radiata* Wilczek) seedlings grown in complete darkness at 26 C for 2.5 to 3 days, and used in all experiments.

Chemicals. Indole-3-acetic acid (IAA), kinetin (6-furfurylaminpurine), 1-aminocyclopropane-1-carboxylic acid (ACC), aminoethoxyvinylglycine (AVG), trifluoperazine (TEP), vcrmapamil, were purchased from Sigma Chemical Co (USA).

Determination of ethylene production. Ten hypocotyl segments were incubated in 2 ml of 50 mM K-phosphate buffcr (pH 6.8) containing 2% sucrosc, 50 μg/ml chloramphcnicol and test chemicals in a 25 ml Erlenmyer flask sealed with a silcone stopper. Thcy were incubated in total darkness at 26 C for appropriate periods, after which a gas sample (1 ml) was withdrawn from the flask with a hypodermic syringe for ethylene determination with a gas chromatograph (Shimadzu GC-3BF).

Assay of EFE activityy. *In vivo* EFE activity was assayed according to a method (Kim nd Kang, 1987) modified from that originalluy developed by Hoffman and yang (1982). Tissue segments pretreated with test substances were thoroughly washed with distilled water and incubated for 2 h in buffer containing 5 μM AVG to block ACC synthesis. The segments wcrc then vaccum infiltrated with 1 mM ACC for 1 h and washed again with distilled water. Ethylene formed by these ACC-preloaded segments represents in viov EFE activity.

RESULTS AND DISCUSSION

Ethylene production from mungbean hypocotyl segments induced by either IAA or kinetin was further promoted by calcium added in the incubation medium (Fig. 1). Calcium treatment, however, was not as effective on the "basal" (i.e. without hormonal induction) rate of ethylene production. Time course data on the calcium promotion of ethylene production from auxin- and kinetin-treated segments clearly show that the calcium effect became manifest with about 6 h of lag period in kinetin-induced ethylenee production while calcium treatment already resulted in substantial increase in ethylene production at 6 h from segments incubated with IAA (Fig. 2). These results indicate that mechanisms by which auxin and kinetin induce ethylene biosynthesis are distinct from each other, and thus that calcium interacts with these two hormones on separate events. The well-established promotion of ethylene biosynthesis by auxin involves specific induction of ACC synthase, the enzyme responsible for the conversion of S-adenosylmethionine (SAM) to ACC (Yoshii *et al.*, 1980; Nakagawa *et al.*, 1988). On the contrary, mechanism of kinetin-induced ethylene production is not clearly understood. Lau and Yang (1975) reported that kinetin promoted auxin-induced ethylene production synergistically, and suggested that the kinetin effect was brought about by increased level of free IAA through suppression by kinetin of IAA conjugation. Imaseki *et al.* (1975) showed that cytokinin action to increase ethylene production was independent of auxin action, but cytokinin enables tissues to respond with a higher sensitivity to auxin. Since cytokinin promoted ethylene production

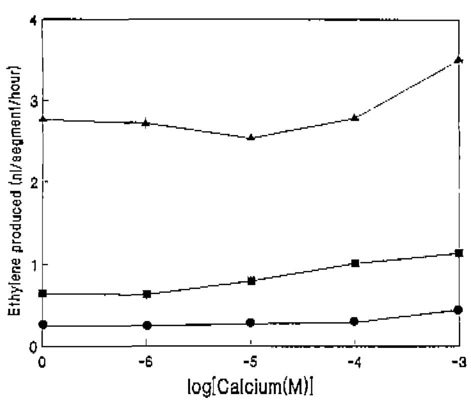

Fig. 1. Ethylene produced from mungbean hypocotyl segments incubated for 12 h with various concentrations of calcium chloride in the absence (circles) and presence of 5 μM kinetin (squares) or auxin (triangles).

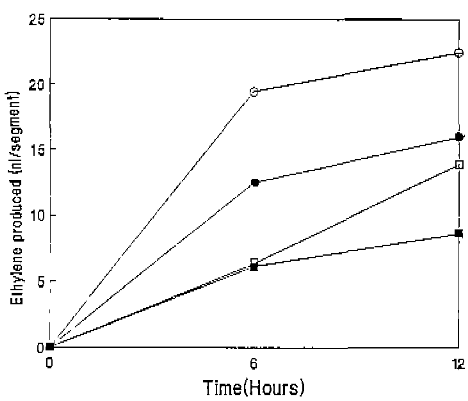

Fig. 2. Ethylene production from hypocotyl segments incubated with 5 μM IAA (circles) or kinetin (squares) in the absence (closed symbols) or presence (open symbols) of 1mM calcium chloride.

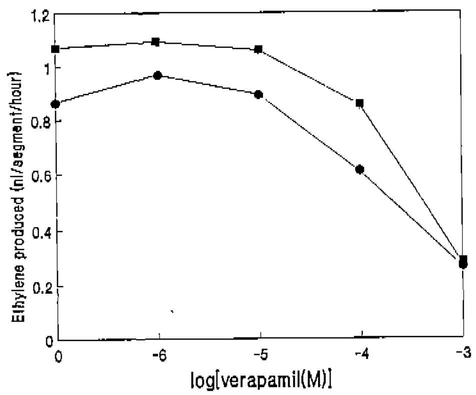

Fig. 3. Effect of various concentrations of verapamil on ethylene production from hypocotyl segments treated with 5 μM IAA in the absence (circles) or presence (squares) of 1 mM calcium chloride.

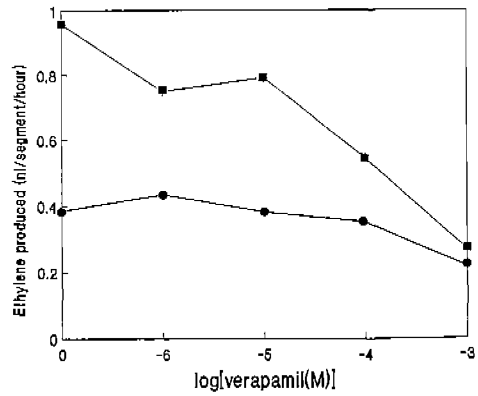

Fig. 4. Effect of variouis concentrations of verapamil on ethylene production from hypocotyl segments treated with 5 μM kinetin in the absence (circles) or presence (squares) of 1 mM calcium chloride.

without a concomitant increase in the ACC level, Yoshii and Imaseki (1981) speculated that cytokinin may increase the availability of ACC at the ethylene synthetic site. In the induction of phenylalanine ammonialyase, auxin and cytokinin were suggested to act on separate events that lead to the increase in the enzyyme (Bevan and Northcote, 1979). In view of the strong likelihood that auxin and cytokinin act separately in the ethylene synthetic system, and of the fact that clcium action at these two separate sites have different kinetice (Fig. 2), it is implied that calcium has dual actions to stimulate ethylene biosynthesis in the system.

The possibility of the calcium action(s) being mediated through calmodulin was explored using a specific inhibitor of calmodulin action, TFP. Many, but not all, calcium regulated cellular responses can be processed when intracellular calcium binds specifically to calmodulin (Cheung, 1980; Babu *et al.*, 1985), and specific calmodulin antagonists have been reported to inhibit such diverse calcium-regulated processes in plants as bud formation in *Funaria*(Saunders and Hdepler, 1983), betacyanin synthesis in *Amaranthus tricolar*(Elliot, 1983; Ellioot *et al.*, 1983) and cell elongatioon in coleoptiles (Raghothama *et al.*, 1985). Data illustrated in Fig. 3 indicate that auxin-induced ethylene production was substantially inhibited by TFP, and that the inhibitory effect of TFP is abolished by a high concentration of external calcium in the medium. However, it is shown in Fig. 4 that TFP exerts no appreciable effect on kinetin-treated tissues regardless of externally applied calcium. These results suggest that the calcium promotion of auxin-induced ethylene production is mediated through calmodulin whereas there is no such involvement of calmodulin in the calcium action on kinetin-induced ethylene production, and support the notion that calcium has dual functions in auxin- and kinetin-induced ethylene biosynthesis.

It is shown in Fig. 5 that treatment of segments with verapamil, which is knowwn to block

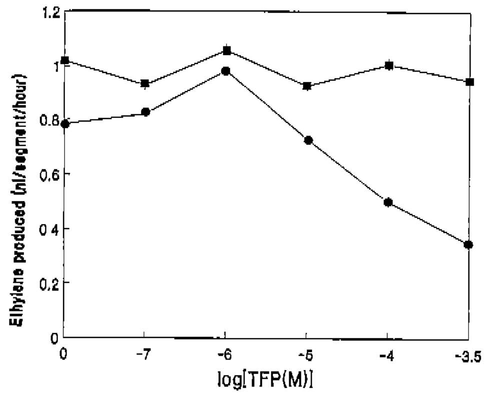

Fig. 5. Effect of various concentrations of TFP on ethylene production from segments treated with 5 μM IAA in the absence (circles) or presence (squares) of 1 mM calcium chloride.

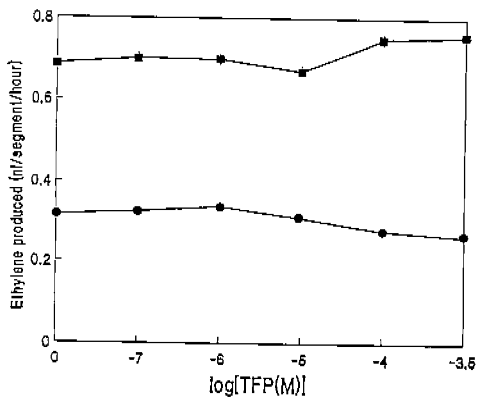

Fig. 6. Effect of various concentrations of TFP on ethylene production from segments treated with 5 μM kinetin in the absence (circles) or presence (squares) of 1 mM calcium chlooride.

Table 1. *In vivo* EFE activity of mung bean hypocotyl segments pretreated for 6hr with auxin, kinetin, and/or $CaCl_2$

$CaCl_2$(mM)	Relative EFF Activity (% of Control)		
	–	IAA (5 μM)	Kinetin (5 μM)
0	100	100	106
1	134	136	160

the calcium channel on the membrane (Lee and Tsien, 1983), resulted in inhibition of auxin-induced ethylene production both in the presence and absence of applied calcium added in the incubation medium. Ther verapamil effect in th absence of added calcium imply that the concentration of calcium in the free space, which is known to be more than thousand times higher than that in the cytosol (Marme, 1983; Hepler nd Wayne, 1985) plays a significant role in the regulation of the cytosolic calcium level. Ethylene production from segments incubated with kinetin, however, was not inhibited to any appreciable extent by verapamil unless added calcium is present in the incubation medium (Fig. 6).

The final step in the sequence of reactions leading to ethylene formation is catalyzed by the enzyme, EFE (Hoffman *et al.*, 1982). Unlike ACC synthase which is inducible, EFE is a constitutive enzyme, and is believed to be membrane-bound (Apelbaum *et al.*, 1981; Mayne and Kende, 1986). Table 1 presents data on EFE activityy of tissues treated with calcium in combination with auxin and kinetin. The data indicate that calcium treatment alone resulted in increased enzyme activity. Neither auxin nor kinetin when applied alone could influence the enzyme activity, but kinetin further increased the enzyyme activity in the presence of calcium whereas auxin did not. In view of the well established action of calcium to support the membrane integrity, and of EFE activity in close association with the membrane, calcium action on ethylene biosynthesis could, at least in part, contains involvement of EFE. It is reported that tissue deprived of calcium had a reduced EFE activity and application of calcium to the tissue would increase the enzyme activity (Moon and Lee, in press). Normally conversion of SAM to ACC catalyzed by ACC synthase constitutes a rate-limiting step in the biosynthetic pathway of ethylene, but EFE-catalyzed ethylene formation from ACC can also function as a minor controlling step in the ethylene synthesizing system. This is illustrated by inhibition of ethylene biosynthesis by osmotic shock (Imaseki and Watanabe, 1978), Triton X-100 or n-propylgallate (Apelbaum *et al.*, 1981a, b) and cobalt ions (Yu *et al.*, 1979). The results of the present work support the idea that calcium on one hand promote auxin-induced ACC synthesis with possible involement of calmodulin, and on the other, stimulate conversion of ACC to ethylene in assoociation with cytokinin action. Probably the latter might constitute a direct action of calcium (i.e. without calmodulin mediation) on the membrane.

摘 要

칼슘은 녹두 하배축 절편에서 오옥신이나 사이토키닌에 의하여 촉진된 에틸렌 생합성을 증가 시켰다. 칼슘에 의한 에틸렌 생성촉진은 키네틴에서 보다 오옥신에 처리한 조직에서 더욱 빨리 발현되었다. 오옥신에 의한 에틸렌 생합성은 calmodulin억제제 TFP에 의하여 억제 되었으며 이 억제는 배양액의 칼슘에 의하여 회복되었으나 키네틴에 의한 에틸렌 생성은 TFP에 의하여 영향을 받지 아니 하였다. 칼슘 channel blocker인 verapamil은 오옥신에의한 에틸렌 생성을 배양액의 칼슘의 유무에 상관없이 억제시켰으나 키네틴에 의한 에틸렌 생성은 배양액에 칼슘이 없을때는 억제효과를 나타내지 못하였다. EFE 효소활성은 칼슘에 의하여 촉진 되었으며 오옥신이나 키네틴은 이 효소에 영향을 주지 아니하였으나 키네틴은 오옥신과는 달리 칼슘에 의하여 증가된 EFE활성에 상승적인 효과를 보여주었다.

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