

Characterization of Carbon Dioxide Stress-Induced Ethylene Biosynthesis in Cucumber (*Cucumis sativus* L.) Fruit

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In the present study, we have investigated the mechanism through which carbon dioxide induces ethylene biosynthesis in cucumber (*Cucumis sativus* L.) fruit. A series of inhibitors were tested in order to determine the involvement of de novo protein synthesis, new mRNA synthesis, protein kinase activity, and phosphoprotein phosphatase activity in carbon dioxide stress-induced ethylene biosynthesis. Carbon dioxide stimulated ethylene production through the induction of both 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase activities. Induction of ACC synthase activity led to accumulation of ACC. Cycloheximide blocked carbon dioxide stress-induced ethylene biosynthesis while cordycepin had no effect. Dibucaine and 6-dimethylaminopurine, inhibitors of protein kinases, blocked carbon dioxide stress-induced increases in ethylene production, ACC synthase activity, and ACC content but not the increase in ACC oxidase activity. Cantharidin, a potent inhibitor of protein phosphatase type 1 and type 2A, stimulated carbon dioxide stress-induced ethylene production, ACC synthase activity, and ACC accumulation but had no additional effect on carbon dioxide stress-induced ACC oxidase activity. In the presence of dibucaine or cycloheximide, the stimulation of carbon dioxide stress-induced ethylene biosynthesis by cantharidin was blocked. These data suggest that carbon dioxide stress-induced ethylene biosynthesis may be regulated posttranscriptionally and that the induction involves the synthesis of novel protein(s) in the cytosol. The results further identify protein phosphorylation and dephosphorylation as requirements in one or more of the steps involved in the carbon dioxide signal transduction pathway that leads to induction of ethylene biosynthesis and, in particular to the induction of ACC synthase, presumably the key enzyme in the ethylene biosynthetic pathway.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AdoMet, S-adenosyl-L-methionine; CHX, cycloheximide; CSI, carbon dioxide stress-induced; DMAP, 6-dimethylaminopurine; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; PLP, pyridoxal-5'-phosphate; PP-1, protein phosphatase type 1; PP-2A, protein phosphatase type 2A.

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Ethylene, one of the simplest organic molecules with biological activity, is a plant hormone that regulates many aspects of plant growth, development, and senescence (Yang and Hoffman 1984, Mattoo and White 1991, Abeles et al. 1992, Mathooko 1996). The biosynthetic pathway of ethylene in higher plants is well defined (Yang and Hoffman 1984, Mathooko 1996). The rate-limiting steps in this pathway in most vegetative tissues are reactions catalyzed by 1-aminocyclopropane-1-carboxylate (ACC) synthase (*S*-adenosyl-L-methionine methylthioadenosine lyase, EC 4.4.1.14), which converts *S*-adenosyl-L-methionine (AdoMet) to ACC, and ACC oxidase (EC 1.4.3), which converts ACC to ethylene (Yang and Hoffman 1984, Kende 1993, Mathooko 1996). These enzymes are developmentally regulated and expressed in response to diverse inducers (Yang and Hoffman 1984, Abeles et al. 1992, Mathooko 1995).

The regulation of ethylene biosynthesis by carbon dioxide has been extensively studied (for review see Mathooko 1996). Elevated carbon dioxide may have mild to serious toxic effects on plant tissues (Abeles et al. 1992, Mathooko 1996). Therefore, subjecting a cultivar of a given commodity to carbon dioxide levels above its tolerance level in a specific temperature-time combination can result in stress to the living plant tissue, which is manifested in various symptoms, such as increased ethylene production (Kubo et al. 1990, Mathooko et al. 1995, Mathooko 1996) among other physiological responses. The physiological role of stress-induced ethylene is not known (Abeles et al. 1992); however, in some cases, the increased level of ethylene may cause stress or induce acclimation processes (Abeles et al. 1992, Morgan and Drew 1997). The mechanism underlying the carbon dioxide signal transduction leading to induction of ethylene biosynthesis has remained unexplored. A multitude of factors such as intracellular pH, protein phosphorylation and dephosphorylation, Ca²⁺ influx from the extracellular space and new mRNA synthesis may participate in the carbon dioxide signal transduction during enhanced ethylene biosynthetic activity.

Intercellular signals and responses to external stimuli

may occur in plants and plant tissues through a reversible protein phosphorylation cascade mediated by protein kinases and protein phosphatases (Ranjeva and Boudet 1987). Indeed, biochemical studies have revealed the roles of some protein kinases and protein phosphatases in signal transduction and hormonal regulation (Stone and Walker 1995, Smith and Walker 1996). Spanu et al. (1994) demonstrated that protein phosphorylation and dephosphorylation are involved in the regulation of elicitor-induced ACC synthase, not by regulating the catalytic activity itself but by controlling the rate of turnover of the enzyme. Inhibitors of protein kinases such as K-252a and staurosporine inhibit elicitor-induced ethylene biosynthesis (Grosskopf et al. 1990, Felix et al. 1991a, Spanu et al. 1994), while inhibitors of protein phosphatase type 1 (PP-1) and type 2A (PP-2A) such as calyculin A can mimic elicitors with respect to induction of ACC synthase and other responses (Felix et al. 1994) and further stimulate elicitor-induced ACC synthase activity (Spanu et al. 1994). These findings indicate that protein phosphorylation plays a central role in the regulation of ethylene biosynthesis, and in particular of ACC synthase. Based on these previous observations, we envisaged that protein phosphorylation and dephosphorylation may play leading roles in carbon dioxide stress-induced (CSI) ethylene biosynthesis.

The use of protein kinase and protein phosphatase inhibitors provides a very powerful approach for the initial assessment of the role of protein phosphorylation and dephosphorylation in controlling numerous cellular events, including signal transduction (Smith and Walker 1996). Therefore, in order to gain more information about the factors involved in the carbon dioxide signal transduction pathway leading to induction of ethylene biosynthesis, we have investigated the effects of cordycepin, an inhibitor of transcription, cycloheximide (CHX), an inhibitor of translation, dibucaine and 6-dimethylaminopurine (DMAP), inhibitors of protein kinases (Mizuno 1994, Comolli et al. 1996), and cantharidin, an inhibitor of PP-1 and PP-2A (Li and Casida 1992, Li et al. 1993, MacKintosh and MacKintosh 1994). Here we show that carbon dioxide stimulates ethylene production by inducing both ACC synthase and ACC oxidase activities and that, while this induction requires de novo protein synthesis, it does not require new mRNA synthesis. We further show that protein phosphorylation and dephosphorylation are involved in the carbon dioxide signaling cascade leading to induction of ACC synthase and not ACC oxidase.

Materials and Methods

Plant material and gas treatment—Freshly harvested greenhouse-grown cucumber (*Cucumis sativus* L. cv. Sharp 1) fruits at commercial maturity (10 to 14 days after anthesis) were obtained from a commercial supplier in Okayama City, Japan. The fruits were sorted with respect to defects, maturity, and uniformity of

shape and size. To determine the effect of carbon dioxide on ethylene biosynthesis, whole fruits were put in 5.5-liter plastic containers and treated at 25°C in a continuous flow-through gas system with either humidified air (control) or with a humidified gas mixture consisting of 80% carbon dioxide and 20% oxygen. To obtain the desired gas mixture, the flow rates of carbon dioxide and oxygen from each of the high pressure gas cylinders were regulated using an electrical flow rate controller (SCEU-1, SEC Inc.). The gas flow rates were maintained at 100 ml min⁻¹. Ethylene production rate was determined from intact fruits; ACC synthase activity, ACC content and ACC oxidase activity were measured in the skin tissue (Wang and Adams 1982, Inaba et al. 1991, Mathooko et al. 1995).

Inhibitors—The inhibitor solutions were prepared as follows: CHX (Sigma), an inhibitor of nucleocytoplasmic protein synthesis, and cordycepin (Sigma), an inhibitor of mRNA synthesis, were dissolved in distilled water at concentrations of 500 μM and 100 μM, respectively. Cantharidin (Sigma), an inhibitor of PP-1 and PP-2A (Li and Casida 1992, Li et al. 1993, MacKintosh and MacKintosh 1994), was dissolved in 0.2% dimethyl sulfoxide (DMSO) at a concentration of 200 μM, and dilutions were prepared in distilled water. DMAP (Sigma), an inhibitor of protein kinases (Mizuno 1994, Comolli et al. 1996), was dissolved in 0.6% DMSO at a concentration of 1 mM. Dibucaine (Sigma), an inhibitor of protein kinases (Mizuno 1994), was dissolved in 1% polyoxyethylene sorbitan monolaurate (Tween 20) at a concentration of 1 mM.

Application of inhibitors—Fruit segments (10 cm long) were cut from the central part of the fruit and soaked in solutions containing the various inhibitors. Such cutting does not induce wound ethylene production (Inaba et al. 1991, Mathooko et al. 1995). The fruit segments were vacuum-infiltrated at 400 mm Hg for 1 min to facilitate absorption of the solutions by the fruit tissue. Control fruits for CHX and cordycepin treatments were infiltrated with distilled water; control fruits for cantharidin and DMAP treatments were infiltrated with 0.2% and 0.6% DMSO solutions, respectively. These DMSO concentrations had no effect on ethylene production, as earlier reported (Kim et al. 1997). Controls for dibucaine treatment were infiltrated with 1% Tween 20 solution. The fruit segments were then treated with humidified air or a humidified gas mixture consisting of 80% carbon dioxide and 20% oxygen as described above.

Ethylene production—Fruit samples were enclosed in 1.5-liter plastic containers fitted with silicon stoppers for gas sampling and incubated at 25°C for 1 h. A 1-ml gas sample was withdrawn from the containers using a gas-tight hypodermic syringe. Ethylene concentration in the headspace gas sample was assayed by injecting the gas sample into a Shimadzu gas chromatograph (Model GC-4CM, Shimadzu Corp., Kyoto, Japan) equipped with an activated alumina column and a flame ionization detector.

Extraction and assay of ACC synthase activity—The extraction and assay of ACC synthase activity was performed according to Boller et al. (1979) with slight modifications as previously described (Mathooko et al. 1993). Five grams of tissue were homogenized in 10 ml of extraction medium consisting of 0.5 M potassium phosphate (pH 8.5), 5 μM pyridoxal-5'-phosphate (PLP), and 5 mM dithiothreitol (DTT) in the presence of 5% polyvinylpyrrolidone. After centrifugation of the homogenate at 10,000 × g for 30 min, the supernatant was passed through a 0.45 μm membrane filter before loading onto prepacked Sephadex G-25 columns (Pharmacia LKB, Uppsala, Sweden) previously equilibrated with 100 mM potassium phosphate buffer solution (pH 8.5) containing 5 μM PLP and 5 mM DTT. The columns were eluted with

the same buffer solution. The resulting protein fractions, free of low molecular weight compounds including ACC, were collected and used in the enzyme assay. All steps in the enzyme extraction were performed at 0–4°C. ACC synthase activity was assayed in a reaction mixture containing 1 ml of 500 μ M AdoMet and 2 ml of the enzyme preparation. After incubation of the reaction mixture at 30°C for 30 min, the ACC formed from AdoMet was assayed by the method of Lizada and Yang (1979), which is based on the chemical degradation of ACC to ethylene. ACC synthase activity was expressed as the amount of ACC (nmol) produced per mg protein per hour.

Extraction and assay of ACC content—ACC in the fruit samples was extracted in 80% ethanol as previously described (Mathooko et al. 1993). The ACC content in the extracts was estimated by the method of Lizada and Yang (1979) as mentioned above.

ACC oxidase assay—ACC oxidase activity was assayed *in vivo* by measuring the capability of the tissue to convert administered ACC to ethylene. Two grams of tissue was put into 40-ml Erlenmeyer flasks containing 2 ml of an incubation solution consisting of 1 ml of 5 mM ACC and 1 ml of 1 mM CHX in 50 mM potassium phosphate buffer (pH 5.3). CHX was included to inhibit new enzyme synthesis. The flasks were vacuum-infiltrated to facilitate absorption of the solution by the tissue and incubated at 30°C for 1 h. The ethylene formed was then determined as described above. ACC oxidase activity was expressed as ethylene (nl) produced per gram fresh weight per hour.

Protein assay—Protein content in the ACC synthase enzyme extracts was estimated by the protein-dye binding method of Bradford (1976) using a Bio Rad Protein Assay kit and bovine serum albumin as a standard.

Results

Kinetics of induction of ethylene biosynthesis by carbon dioxide—Carbon dioxide has previously been shown to induce ethylene production in several plant tissues (Kubo et al. 1990). Elevated carbon dioxide stimulated ethylene production in cucumber fruits (Fig. 1A). This stimulation was due to induction of the activities of both ACC synthase (Fig. 1B) and ACC oxidase (Fig. 1C). The induction of ethylene biosynthesis by carbon dioxide occurred after a lag period of 3 h. Further, this induction of ethylene biosynthesis by carbon dioxide required continuous presence of carbon dioxide, because upon withdrawal of the gas, ethylene biosynthesis returned to near control level.

Effects of inhibitors of translation and transcription—The apparent induction of ethylene biosynthesis by carbon dioxide could reflect a covalent modification, protein turnover, or a tight-binding activator/inhibitor. To begin to distinguish among these possibilities, we infiltrated fruits with the protein synthesis inhibitor CHX prior to treatment with air or carbon dioxide. CHX blocks translation on cytoplasmic ribosomes and inhibits the elongation of polypeptide chains in the cytosol of eukaryotic cells; it is widely used for inhibition of protein synthesis. CHX blocked CSI increases in ethylene production, activities of ACC synthase and ACC oxidase, and ACC content (Table 1). Further, the induction of ACC synthase and ACC oxidase by vari-

ous stimuli could be under transcriptional or posttranscriptional mechanisms of regulation. Therefore, in order to understand the mechanism through which CSI ACC synthase and ACC oxidase are regulated, we infiltrated fruits with 100 μ M of cordycepin, an inhibitor of transcription, prior to treatment with air or carbon dioxide. Treatment of fruits with cordycepin had no effect on CSI ethylene biosyn-

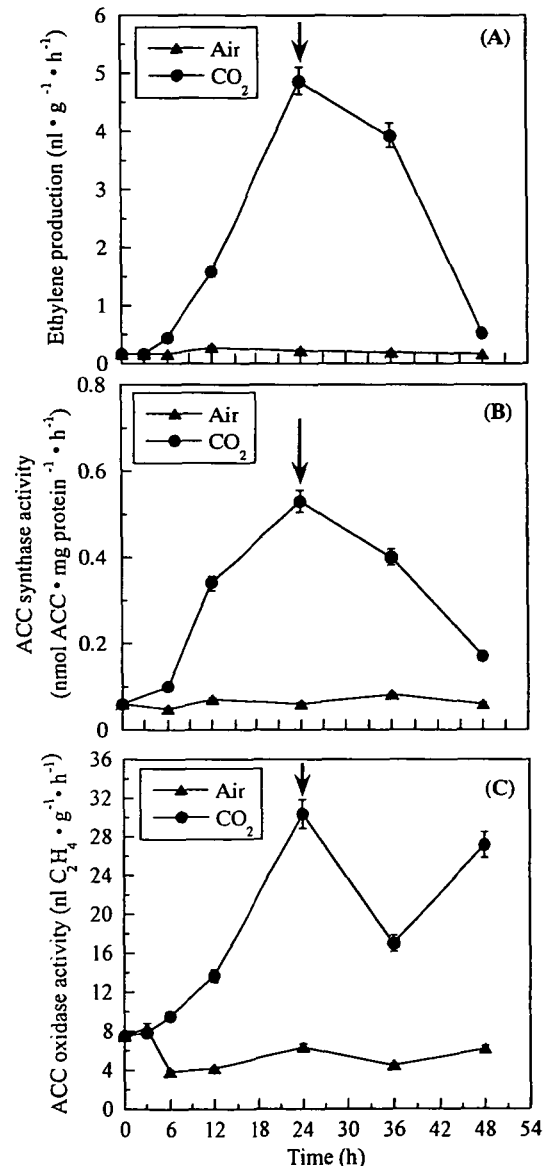


Fig. 1 Time course of carbon-dioxide-stress-induced ethylene production (A), ACC synthase activity (B), and ACC oxidase activity (C). Fruits were treated at 25°C in a continuous flow-through gas system with humidified air (control) or a humidified gas mixture consisting of 80% carbon dioxide and 20% oxygen. The arrow indicates the time of transfer of the fruits from carbon dioxide-enriched atmosphere to air. The vertical bars represent the SE of the mean of three replications. When absent, the SE bars fall within the dimensions of the symbol.

Table 1 Effects of cycloheximide and cordycepin on carbon dioxide stress-induced ethylene biosynthesis in cucumber fruits

Inhibitor	Treatment	Ethylene production (nl g ⁻¹ h ⁻¹)	ACC synthase activity (nmol ACC (mg protein) ⁻¹ h ⁻¹)	ACC oxidase activity (nl C ₂ H ₄ g ⁻¹ h ⁻¹)	ACC content (nmol g ⁻¹)
Cycloheximide	Air	0.08 ± 0.01	0.02 ± 0.00	10.43 ± 1.27	0.27 ± 0.04
	Air + Cycloheximide	0.87 ± 0.04	0.10 ± 0.01	9.47 ± 0.98	1.37 ± 0.28
	CO ₂	7.64 ± 0.79	0.81 ± 0.14	27.54 ± 2.76	12.92 ± 1.80
	CO ₂ + Cycloheximide	1.48 ± 0.18	0.04 ± 0.01	11.97 ± 1.27	1.25 ± 0.43
Cordycepin	Air	0.03 ± 0.00	0.04 ± 0.00	13.20 ± 1.30	0.35 ± 0.09
	Air + Cordycepin	0.03 ± 0.00	0.03 ± 0.00	17.29 ± 2.01	0.41 ± 0.05
	CO ₂	8.09 ± 0.15	0.51 ± 0.02	28.82 ± 2.36	15.86 ± 2.71
	CO ₂ + Cordycepin	7.79 ± 0.81	0.52 ± 0.03	32.05 ± 3.92	17.09 ± 2.42

Fruit segments (10 cm long) were vacuum-infiltrated with distilled water (controls) or with solutions containing 500 μM cycloheximide or 100 μM cordycepin. They were then treated at 25°C in a continuous flow-through gas system with humidified air or a humidified gas mixture consisting of 80% carbon dioxide and 20% oxygen for 24 h. The values are means ± SE of three replications.

thesis with respect to ethylene production, ACC synthase activity, ACC content, or ACC oxidase activity (Table 1).

Effects of protein kinase inhibitors—Protein phosphorylation plays a major role in signal transduction. In the present study, we used DMAP and dibucaine, specific inhibitors of protein kinases, to investigate the involvement of protein phosphorylation in carbon dioxide signal transduction. Both DMAP and dibucaine inhibited CSI ethylene production, ACC synthase activity, and hence ACC accumulation (Table 2). However, these protein kinase inhibitors had no effect on the ACC oxidase activity induced by carbon dioxide. Therefore, they seem to regulate CSI ethylene biosynthesis through their effects on ACC synthase.

Effect of protein phosphatase inhibitor—Processes that are reversibly controlled by protein phosphorylation require not only a protein kinase, but also a protein phosphatase (Hunter 1995). Therefore, one would expect a decrease in phosphorylation of ACC synthase in tissues treated with DMAP or dibucaine; inhibitors of protein phosphatase should have the opposite effect. The fact that DMAP and dibucaine blocked the carbon dioxide induction of ethylene biosynthesis and, in particular, of ACC synthase activity led us to imagine that the effects of DMAP and dibucaine may be connected to protein phosphatases. We, therefore, investigated the effect of cantharidin (a simple terpene produced by meloid blister beetles such as *Lytta vesicifera*), a

Table 2 Effects of 6-dimethylaminopurine (DMAP) and dibucaine on carbon dioxide stress-induced ethylene biosynthesis in cucumber fruits

Inhibitor	Treatment	Ethylene production (nl g ⁻¹ h ⁻¹)	ACC synthase activity (nmol ACC (mg protein) ⁻¹ h ⁻¹)	ACC oxidase activity (nl C ₂ H ₄ g ⁻¹ h ⁻¹)	ACC content (nmol g ⁻¹)
DMAP	Air	0.08 ± 0.01	0.01 ± 0.00	10.23 ± 1.36	0.28 ± 0.05
	Air + DMAP	0.30 ± 0.01	0.03 ± 0.01	16.76 ± 1.52	0.30 ± 0.05
	CO ₂	4.52 ± 0.12	0.41 ± 0.03	29.83 ± 0.69	12.69 ± 1.61
	CO ₂ + DMAP	1.57 ± 0.24	0.09 ± 0.01	30.17 ± 2.76	3.46 ± 1.54
Dibucaine	Air	0.05 ± 0.01	0.03 ± 0.00	10.31 ± 1.72	0.23 ± 0.07
	Air + Dibucaine	0.08 ± 0.02	0.04 ± 0.00	9.81 ± 0.92	0.31 ± 0.07
	CO ₂	6.53 ± 1.25	0.47 ± 0.11	25.62 ± 3.70	14.81 ± 2.17
	CO ₂ + Dibucaine	0.81 ± 0.07	0.07 ± 0.01	22.15 ± 2.97	1.85 ± 0.18

Fruit segments (10 cm long) were vacuum-infiltrated with a 0.6% DMSO solution (DMAP controls) or a solution containing 1 mM DMAP dissolved in 0.6% DMSO or 1% Tween 20 solution (dibucaine controls) or a solution containing 1 mM dibucaine in 1% Tween 20 solution. They were then treated at 25°C in a continuous flow-through gas system with humidified air or a humidified gas mixture consisting of 80% carbon dioxide and 20% oxygen for 24 h. The values are means ± SE of three replications.

Table 3 Effect of cantharidin on carbon dioxide stress-induced ethylene biosynthesis in cucumber fruits

Treatment	Ethylene production (nl g ⁻¹ h ⁻¹)	ACC synthase activity (nmol ACC (mg protein) ⁻¹ h ⁻¹)	ACC oxidase activity (nl C ₂ H ₄ g ⁻¹ h ⁻¹)	ACC content (nmol g ⁻¹)
Air	0.05±0.01	0.02±0.00	10.55±0.92	0.15±0.01
Air + Cantharidin	1.62±0.03	0.05±0.01	17.17±1.08	0.52±0.11
CO ₂	5.86±0.75	0.34±0.07	28.99±2.07	8.85±0.38
CO ₂ + Cantharidin	15.63±1.74	0.90±0.09	29.47±1.64	22.91±0.72

Fruit segments (10 cm long) were vacuum infiltrated with a 0.2% DMSO solution (controls) or a solution containing 100 μM cantharidin dissolved in 0.2% DMSO. They were then treated at 25°C in a continuous flow-through gas system with humidified air or a humidified gas mixture consisting of 80% carbon dioxide and 20% oxygen for 24 h. The values are means ± SE of three replications.

relatively new inhibitor of PP-1 and PP-2A (Li and Casida 1992, Li et al. 1993). Initially, we treated the fruits with 100 μM cantharidin followed by treatment with air or carbon dioxide for 24 h in order to examine its effect on CSI ethylene biosynthesis. Cantharidin slightly stimulated ethylene biosynthesis in the control fruits (Table 3). However, when fruits pretreated with cantharidin were treated with carbon dioxide, there were three-fold increases in ethylene production, ACC synthase activity, and ACC accumulation over those caused by treatment with carbon dioxide alone (Table 3). Interestingly, pretreatment of fruits with cantharidin followed by treatment with carbon dioxide did not cause any increase in ACC oxidase activity beyond that caused by carbon dioxide alone. However, in the absence of carbon dioxide, cantharidin caused a slight increase in ACC oxidase activity. This clearly indicates that, like the protein kinase(s), the protein phosphatase(s) involved in the regulation of CSI ethylene biosynthesis is directed towards ACC synthase.

Concentration-dependence of stimulation of CSI ethylene biosynthesis by cantharidin—In order to understand the concentration dependence of cantharidin in the stimulation of CSI ethylene biosynthesis, fruits were treated with

0, 1, 10, 50, 100, or 200 μM of cantharidin prior to treatment with carbon dioxide. The stimulation of CSI ethylene production was noticeable at cantharidin concentrations of 10 μM and above. Thereafter, the stimulation of ethylene production increased with increase in cantharidin concentration (Fig. 2A). The increase in ethylene production was accompanied by increases in ACC synthase activity (Fig. 2B) and ACC content (Fig. 2C). Indeed, it has previously been indicated that relatively high concentrations of cantharidin are required to cause inhibition (MacKintosh and MacKintosh 1994).

Effect of withdrawal of carbon dioxide on cantharidin stimulatory effect—We further investigated whether the stimulation of CSI ethylene biosynthesis by cantharidin requires the continuous presence of carbon dioxide in the storage environment or not. Fruits were infiltrated with 0.2% DMSO (control), 200 μM cantharidin, or 1 mM dibucaine and then treated with carbon dioxide for 24 h. Ethylene production rates were monitored at regular intervals. As expected, treatment of fruits with carbon dioxide induced ethylene production by the fruits, which was further enhanced by pretreatment with cantharidin and inhibited by pretreatment with dibucaine (Fig. 3). Upon withdrawal

Table 4 Effects of dibucaine (DIB) and cycloheximide (CHX) on the stimulation of carbon dioxide stress-induced ethylene biosynthesis in cucumber fruits by cantharidin (CAN)

Treatment	Ethylene production (nl g ⁻¹ h ⁻¹)	ACC synthase activity (nmol ACC (mg protein) ⁻¹ h ⁻¹)	ACC oxidase activity (nl C ₂ H ₄ g ⁻¹ h ⁻¹)	ACC content (nmol g ⁻¹)
CO ₂	5.12±0.90	0.35±0.07	24.81±3.72	8.13±2.15
CO ₂ + CAN	14.75±2.34	0.82±0.15	27.77±2.10	17.82±2.60
CO ₂ + DIB	1.17±0.24	0.06±0.01	28.20±3.18	0.38±0.06
CO ₂ + CHX	1.21±0.15	0.05±0.01	8.17±0.90	0.30±0.07
CO ₂ + CAN + DIB	1.50±0.30	0.05±0.00	30.10±3.50	0.44±0.10
CO ₂ + CAN + CHX	1.88±0.22	0.07±0.01	9.25±1.70	0.47±0.09

Fruit segments (10 cm long) were vacuum-infiltrated with 0.2% DMSO (control) [a control with 1% Tween 20 solution gave a similar trend] or solutions containing 1 mM dibucaine in 1% Tween 20 solution, 500 μM cycloheximide, 200 μM cantharidin separately or in combination as indicated. They were then treated at 25°C in a continuous flow-through gas system with a humidified gas mixture consisting of 80% carbon dioxide and 20% oxygen for 24 h. The values are means ± SE of three replications.

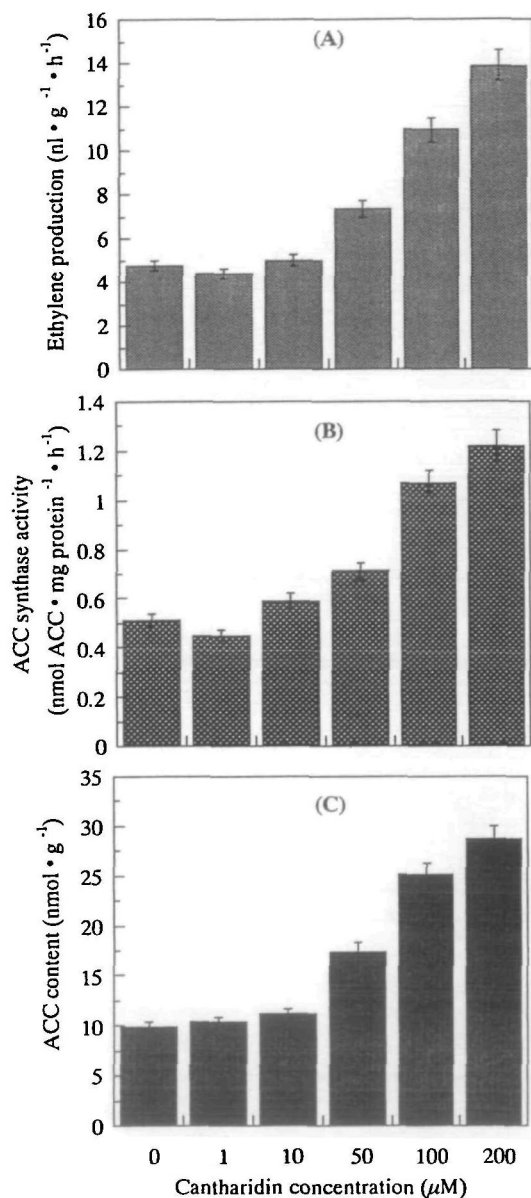


Fig. 2 Effect of cantharidin concentration on carbon-dioxide-stress-induced ethylene production (A), ACC synthase activity (B), and ACC content (C). Fruit segments (10 cm long) were vacuum-infiltrated with 0.2% DMSO (control) or 0.2% DMSO solutions containing the various concentrations of cantharidin and treated at 25°C in a continuous flow-through gas system with a humidified gas mixture consisting of 80% carbon dioxide and 20% oxygen for 24 h. The vertical bars represent the SE of the mean of three replications.

of carbon dioxide from the storage environment, ethylene production in carbon dioxide-treated fruits started to decline; in the fruits pretreated with cantharidin, there was a transient increase in ethylene production followed by a decline. Withdrawal of carbon dioxide had no effect on eth-

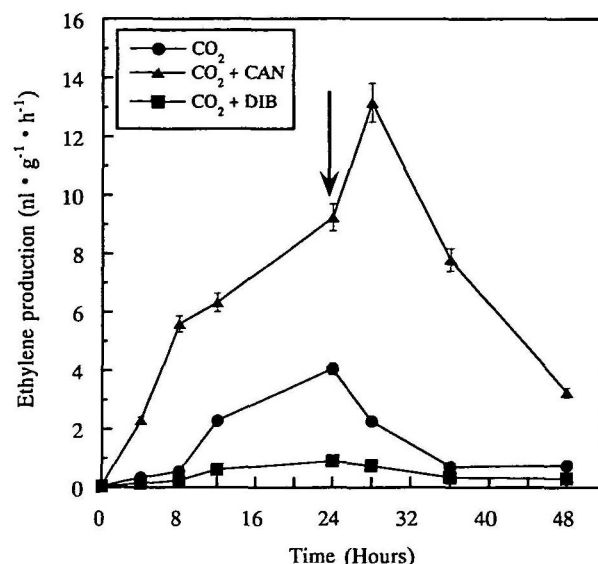


Fig. 3 Time course of carbon-dioxide-stress-induced ethylene production as influenced by cantharidin (CAN) and dibucaine (DIB). Fruit segments (10 cm long) were vacuum-infiltrated with 0.2% DMSO (control), 200 μM CAN, or 1 mM DIB solutions as described in Materials and Methods. They were then treated at 25°C in a continuous flow-through gas system with a humidified gas mixture consisting of 80% carbon dioxide and 20% oxygen. The arrow indicates the time of transfer of the fruit segments from carbon dioxide-enriched atmosphere to air. The vertical bars represent the SE of the mean of three replications. When absent, the SE bars fall within the dimensions of the symbol.

ylene production by fruits pretreated with dibucaine.

Effects of dibucaine and CHX on cantharidin-stimulated ethylene biosynthesis—From the results presented above (Table 2, 3), it is evident that the protein kinase and protein phosphatase inhibitors have opposite effects on ACC synthase activity. This raises the question of whether the carbon dioxide signal transduction events requiring protein kinase(s) and protein phosphatase(s) act through the same pathway or not. Further, it is not known whether the stimulation of CSI ethylene biosynthesis by cantharidin has an absolute requirement for protein synthesis or not. To understand these issues, fruits were infiltrated with CHX or dibucaine prior to treatment with cantharidin and/or carbon dioxide. As shown in Table 4, infiltration of fruits with dibucaine blocked the stimulatory effects of cantharidin on CSI ethylene production, ACC synthase activity, and ACC accumulation but not on ACC oxidase activity. Infiltration of fruits with CHX blocked the stimulatory effect of cantharidin on CSI ethylene production, ACC synthase activity, ACC accumulation, and ACC oxidase activity.

Discussion

In most cases where ethylene production is increased

by various stimuli, the response is mainly due to the induction of ACC synthase, presumably the rate-limiting enzyme in the ethylene biosynthetic pathway, and, in some cases, to the induction of ACC oxidase (Kende 1993). Our results show that this is also the case with CSI ethylene biosynthesis and are consistent with our previous observations (Kubo et al. 1990, Mathooko et al. 1995). The response was due to the presence of carbon dioxide, since withdrawal of the gas resulted in reduced ethylene producing capacity. A similar phenomenon was observed in several horticultural commodities (Kubo et al. 1990). Felix et al. (1991a) observed that, in suspension-cultured tomato cells treated with an elicitor in which ethylene production and ACC synthase activity were stimulated, removal of the elicitor caused a rapid cessation of the increase in ACC synthase activity, suggesting that continuous presence of the stimulus was necessary for the response. As with other mild toxicants, the ability of carbon dioxide to promote ethylene biosynthetic activity may be due to a stress effect (Abeles et al. 1992).

The relative roles of transcription and translation in stress protein synthesis are not understood (Abeles et al. 1992). Experiments with inhibitors of protein and mRNA syntheses suggest that stress results in the translation of preformed mRNAs (Abeles et al. 1992). The blocking of carbon dioxide stress induction of ACC synthase and ACC oxidase and consequently of ethylene production by CHX indicates that the carbon dioxide response is not a consequence of activation of preexisting enzymes, as suggested by Pesis et al. (1994), but is rather due to *de novo* synthesis of the enzymes or other proteins required for the induction. This inhibition further suggests that the protein(s) necessary for the induction of ethylene biosynthesis is either rapidly turned over or not present constitutively and is thus induced following carbon dioxide treatment. Ethylene production induced by many stimuli other than copper ions (Mattoo et al. 1992), including wounding (Kende and Boller 1981), chilling (Wang and Adams 1982), fungal elicitor (Chappell et al. 1984, Felix et al. 1991a), irradiation (Larrigaudiere et al. 1990), and auxin (Kim et al. 1992) have shown an absolute requirement for *de novo* protein synthesis. However, it is not yet known whether the protein step is required for the activation of ACC synthase and ACC oxidase or for their synthesis. It could also involve the inhibition of a rapidly synthesized protein(s), which in turn activates ACC synthase and ACC oxidase rather than affecting the synthesis and/or turnover of these enzymes *per se*. The lack of any effect of cordycepin on CSI ethylene biosynthesis suggests that the transcribed forms of ACC synthase and ACC oxidase are already present and need not be newly transcribed but must be translated following treatment with carbon dioxide. Indeed, in some ethylene-producing systems, transcription of ACC synthase and ACC oxidase genes may not be the only factor regulating

the production of ACC and ethylene; regulatory mechanisms at the posttranscriptional and posttranslational levels may be equally important (Oetiker et al. 1997). These could act at the level of mRNA splicing, in the control of translation, or by modification of the native ACC synthase protein in particular and could include C- and N-terminal processing and covalent modifications such as phosphorylation or alkylation (Oetiker et al. 1997). Consistent with our observation, elicitor-induced increases in ACC synthase activity in parsley cells (Chappell et al. 1984) and tomato suspension-cultured cells (Spanu et al. 1993, 1994) were not blocked by either cordycepin or actinomycin D, both inhibitors of mRNA synthesis. A similar phenomenon has also been observed in petunia stigmas after pollination (Pech et al. 1987), cucumber fruits subjected to chilling stress (Wang and Adams 1982) and cherry tomatoes after gamma irradiation (Larrigaudiere et al. 1990). However, how carbon dioxide activates the preexisting mRNAs for these two enzymes remains to be elucidated.

In plants, evidence for participation of protein phosphorylation in signal transduction has been studied for light signals (Smith and Walker 1996), elicitor response (Felix et al. 1991b, 1994, Spanu et al. 1994), and hormone stimuli such as cytokinins, auxins, and ethylene (Dominov et al. 1992, Raz and Fluhr 1993, Kim et al. 1997). The blocking of CSI ethylene biosynthesis and, in particular, of ACC synthase activity by DMAP and dibucaine indicates that a key protein kinase(s) is required for the signal transduction. A protein kinase inhibitor, K-252a, inhibited the induction of phenylalanine ammonia-lyase activity in soybean suspension-cultured cells (MacKintosh et al. 1994) and ethylene biosynthesis in tomato suspension-cultured cells following treatment with a yeast extract elicitor (Grosskopf et al. 1990, Felix et al. 1991b, Spanu et al. 1994). In elicitor-stimulated tomato cells, K-252a not only prevented the induction of ACC synthase activity by elicitors, but it also promoted its apparent turnover (Spanu et al. 1994). Further, K-252a and staurosporine, another inhibitor of protein kinase, blocked elicitor-induced changes in the pattern of protein phosphorylation (Felix et al. 1991b). Models for signal transduction are generally based on the notion that binding of a signal molecule to its receptor activates a protein kinase, resulting in phosphorylation of proteins involved in signal transduction (Felix et al. 1994). Our results, therefore, indicate that some of the components of the carbon dioxide signaling pathway are protein kinases, suggesting that plant tissues sense carbon dioxide stress signals via a kinase cascade.

Two groups of protein phosphatases, PP-1 and PP-2A, are responsible for the dephosphorylation of phosphoserine and/or phosphothreonine in eukaryotes (Comolli et al. 1996). The stimulation of CSI ACC synthase by cantharidin suggests that ACC synthase induced by carbon dioxide is most active in its phosphorylated state and that

there is a protein phosphatase(s) that dictates ACC synthase activity via its effects on this enzyme's phosphorylation state, thereby increasing its response to carbon dioxide. Therefore, the activities of PP-1 and PP-2A and/or related enzymes play important roles in the regulation of the carbon dioxide stress ethylene-producing system. This suggestion is consistent with reports that elicitor activation of parsley cells and tomato cells causes phosphorylation of some intracellular proteins and dephosphorylation of others (Dietrich et al. 1990, Felix et al. 1991b). Spanu et al. (1994) observed that calyculin A, an inhibitor of PP-1 and PP-2A, caused some stimulation of ethylene biosynthesis in suspension-cultured tomato cells in the absence of an elicitor and accelerated the rate of increase in ACC synthase activity in elicitor-stimulated cells. Calyculin A also slightly stimulated ACC oxidase activity in suspension-cultured cells in the absence of a fungal elicitor, xylanase (Felix et al. 1994), an observation which is consistent with ours. The slight stimulation of ethylene biosynthetic activity by cantharidin in the absence of carbon dioxide is consistent with the hypothesis that the proteins critical for signaling are continually phosphorylated and dephosphorylated even in the absence of the stimuli and that the phosphorylation status of the relevant proteins is low in the basal state because the protein phosphatases involved are more active than the corresponding protein kinases (Felix et al. 1994).

The blocking of the cantharidin stimulatory effect on CSI ethylene biosynthesis by dibucaine suggests that the protein kinase(s) and protein phosphatase(s) involved in carbon dioxide signal transduction act through the same pathway. This may also be taken to indicate that a basal level of protein phosphorylation is necessary for the action of both carbon dioxide and cantharidin (Pshenichkin and Wise 1995). In suspension-cultured tomato cells, calyculin A mimicked the action of an elicitor and induced strong increase in ACC synthase activity; this response was, however, inhibited by K-252a (Felix et al. 1994). In tobacco leaves, pathogen-induced responses induced by okadaic acid, an inhibitor of PP-1 and PP-2A, were blocked by kinase inhibitors (Raz and Fluhr 1993); in soybean cell suspension culture, addition of cantharidin induced phenylalanine ammonia-lyase activity which was largely prevented by K-252a (MacKintosh et al. 1994). The inhibition of the cantharidin stimulatory effect on CSI ethylene biosynthesis by CHX implies that *de novo* protein synthesis is necessary for its action. In soybean cell culture, the induction of phenylalanine ammonia-lyase by okadaic acid was completely blocked by CHX and K-252a (MacKintosh et al. 1994). Further, Spanu et al. (1994) reported that the increase in ACC synthase activity caused by calyculin A in elicitor-stimulated tomato cells was blocked in the presence of CHX, indicating that the effect of these inhibitors on PP-1 and PP-2A is dependent on new protein synthesis in the cytosol.

How the carbon dioxide signal is perceived by the cell and how the signal is transduced to trigger ethylene production is not known. The effects of protein kinase and protein phosphatase inhibitors on enzyme activities are often due to direct activation/inactivation of the enzymes by reversible phosphorylation (Cohen and Cohen 1989). According to this model, it appears, therefore, that phospho-ACC synthase would be the enzymatically active form and dephospho-ACC synthase, the enzymatically inactive form. However, as observed by Spanu et al. (1994) and based on our observation in this study, such a model may not have validity, since in the presence of the protein synthesis inhibitor, CHX, the cantharidin-stimulated increase in CSI ACC synthase activity was blocked, indicating that protein synthesis is needed to sustain the increase in the enzyme activity and that there is no pool of inactive ACC synthase e.g. dephospho-ACC synthase. It is plausible that changes in protein phosphorylation lead to changes in turnover of the enzyme. Based on our results, we concur with Spanu et al. (1994), who proposed that ACC synthase may be the substrate of protein kinases and protein phosphatases and that its dephosphorylated form, although enzymatically active, may be targeted to a process of inactivation. Another line of argument is that the protein phosphatase inhibitor, cantharidin, can cause posttranscriptional modification in the form of C-terminal processing of ACC synthase, a condition which has been shown to lead to hyperactive forms of ACC synthase (Li and Mattoo 1994). At present it is not known whether ACC synthase is phosphorylated or not. Nevertheless, this is the first time, albeit indirectly, that protein phosphorylation and dephosphorylation have been implicated in the carbon dioxide signal transduction pathway that leads to induction of ethylene biosynthesis. However, identification of the specific protein kinase(s) and protein phosphatase(s) and their protein substrates is necessary in order to gain a better understanding of the carbon dioxide signal transduction.

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