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Expression Characteristics of *CS-ACS1, CS-ACS2* and *CS-ACS3*, Three Members of the 1-Aminocyclopropane-1-Carboxylate Synthase Gene Family in Cucumber (*Cucumis sativus* L.) Fruit under Carbon Dioxide Stress

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We investigated the expression pattern of three 1-aminocyclopropane-1-carboxylate (ACC) synthase genes, *CS-ACS1, CS-ACS2* and *CS-ACS3* in cucumber (*Cucumis sativus* L.) fruit under CO_2 stress. CO_2 stress-induced ethylene production paralleled the accumulation of only *CS-ACS1* transcripts which disappeared upon withdrawal of CO_2. Cycloheximide inhibited the CO_2 stress-induced ethylene production but superinduced the accumulation of *CS-ACS1* transcript. At higher concentrations, cycloheximide also induced the accumulation of *CS-ACS2* and *CS-ACS3* transcripts. In the presence of CO_2 and cycloheximide, the accumulation of *CS-ACS2* transcript occurred within 1 h, disappeared after 3 h and increased greatly upon withdrawal of CO_2. Inhibitors of protein kinase and types 1 and 2A protein phosphatases which inhibited and stimulated, respectively, CO_2 stress-induced ethylene production had little effect on the expression of these genes. The results presented here identify *CS-ACS1* as the main ACC synthase gene responsible for the increased ethylene biosynthesis in cucumber fruit under CO_2 stress and suggest that this gene is a primary response gene and its expression is under negative control since it is expressed by treatment with cycloheximide. The results further suggest that the regulation of CO_2 stress-induced ethylene biosynthesis by reversible protein phosphorylation does not result from enhanced ACC synthase transcription.

Key words: ACC synthase — CO_2 stress — Cucumber — *Cucumis sativus* — Ethylene biosynthesis — Gene expression — Signal transduction.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; CHI, cycloheximide; CS-ACS, *Cucumis sativus* ACC synthase; DMAP, 6-dimethylaminopurine; RT-PCR, reverse transcriptase-polymerase chain reaction.

The nucleotide sequences for the *CS-ACS1, CS-ACS2, CS-ACS* and actin genes used in this study have been submitted to the DDBJ, EMBL and GenBank under accession numbers, AB006803, AB006804, AB006805, and AB010922, respectively.

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Ethylene, a major phytohormone, is one of the simplest organic molecules with biological activity and controls many aspects of plant growth, development, and senescence (Yang and Hoffman 1984, Abeles et al. 1992). The rate of ethylene production is usually low in most tissues, but increases drastically at certain developmental stages, including seed germination, leaf abscission, and fruit ripening (Yang and Hoffman 1984, Zarembinski and Theologis 1994, Fluhr and Mattoo 1996). Ethylene production is also induced by various stress conditions among them wounding, temperature fluctuations, drought, anaerobiosis, CO_2, and viral infection (Yang and Hoffman 1984, Abeles et al. 1992, Mathooko 1995, 1996). The enzyme 1-aminocyclopropane-1-carboxylate (ACC) synthase (*S*-adenosyl-L-methionine methylthioadenosine lyase, EC 4.4.1.14) catalyzes the conversion of *S*-adenosyl-L-methionine to ACC, presumably the rate-limiting step in the ethylene biosynthetic pathway (Yang and Hoffman 1984, Kende 1993, Mathooko 1996). The enzyme is encoded by a family of genes and each gene is independently regulated and differentially expressed in response to various signals, thereby providing an ideal system for studying complex signal transduction pathways in relation to growth, development, stress, and senescence (Kende 1993, Zarembinski and Theologis 1994, Fluhr and Mattoo 1996).

The molecular basis for inhibition of ethylene biosynthesis by elevated levels of CO_2 has recently been studied, and it has been demonstrated that this inhibition is due to suppression of the expression of both ACC synthase and ACC oxidase at the mRNA level (Kubo et al. 1995, Gorney and Kader 1996, 1997, Mathooko et al. 1997, Rothan et al. 1997). However, although it has been known for a long time that CO_2 induces ethylene biosynthesis in several plant tissues (Kubo et al. 1990, Mathooko 1996), it is only recently that the biochemical basis was established (Mathooko et al. 1995a, 1998). Moreover, little information is available about the molecular mechanisms by which plant cells sense and respond to CO_2 stress and how the signal leading to induction of ethylene biosynthesis is transmitted to the nucleus. The mechanism(s) underlying the conversion of CO_2 signals into changes in ACC synthase gene expression remains largely unexplored. A mul-

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titude of factors such as mRNA stabilization, reversible protein phosphorylation or cytosolic pH changes may participate in signaling during ACC synthase induction (Mathooko 1996, Mathooko et al. 1998). Based on current evidence, the induction of ACC synthase by various stimuli is controlled by a complex network of independent yet interrelated signaling pathways. Recent evidence suggests that specific sets of protein kinases and phosphatases catalyze reversible phosphorylation steps responsible for induction of ACC synthase by CO_2 (Mathooko et al. 1998). Further, de novo synthesis of ACC synthase, controlled by the rate of transcription of its genes has been widely accepted as the way ethylene production is regulated, and this generalization has been applied to the promotion of ethylene production by stress (Abeles et al. 1992, Morgan and Drew 1997). In addition to this transcriptional control of gene expression, posttranscriptional control seems to play an important role in stress ethylene biosynthesis (Spanu et al. 1994, Morgan and Drew 1997). This is because experiments with inhibitors of protein and mRNA syntheses suggest that stress results in the translation of preformed mRNAs (Abeles et al. 1992).

We are currently interested in elucidating some of the components of the signal transduction pathway(s) involved in CO_2 stress leading to induction of ethylene biosynthesis using both biochemical and molecular biological approaches. Understanding the molecular details of this CO_2 signal transduction pathway(s) may ultimately allow genetic engineering of fruit and vegetable varieties tolerant to high levels of CO_2 during controlled atmosphere storage. The role of ACC synthase genes in cucumber in relation to sex expression has recently been reported (Kamachi et al. 1997, Trebitsh et al. 1997). In our laboratory, DNA sequences of three ACC synthase genes from cucumber fruit, namely *CS-ACS1, CS-ACS2* and *CS-ACS3* have been described (Shiomi et al. 1998). *CS-ACS1* has been shown to be induced by wounding, *CS-ACS2* to be expressed nonspecifically and *CS-ACS3* to be induced by auxin treatment. Isolated genes can be used as molecular probes for elucidating the signal transduction pathway(s) that mediates the induction of ethylene production in plant tissues under stress (Abel et al. 1995). Based on this approach the present study was designed to identify the CO_2 stress-inducible ACC synthase gene(s) among the three genes we have so far isolated and to investigate the roles of protein synthesis, and protein phosphorylation and dephosphorylation in mediating the expression of the three ACC synthase genes in cucumber fruit under CO_2 stress. Here we show that *CS-ACS1* is the main ACC synthase gene responsible for enhanced ethylene biosynthesis in cucumber fruit under CO_2 stress and the accumulation of its transcripts is superinduced by cycloheximide (CHI). Further, in the presence of CHI, CO_2 is able to induce the other two ACC synthase genes. We also demonstrate that although reversible protein phosphorylation regulates CO_2 stress-induced ethylene biosynthesis, these processes might have little or no role in mediating the CO_2 stress leading to the accumulation of ACC synthase transcripts.

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 d 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 and treated at 25°C in a continuous flow-through gas system with either humidified air (control) or a humidified gas mixture consisting of 80% CO_2 and 20% O_2 as previously described (Mathooko et al. 1998). Ethylene production rate was determined from intact fruits and total RNA was extracted from the skin tissue. This is based on our past work which has provided clear evidence that the ethylene production rate from fruits treated with elevated CO_2 closely correlates with the induction of ACC synthase and accumulation of ACC in the skin tissue (Mathooko et al. 1995a, 1998). Samples for RNA extraction were frozen in liquid nitrogen and stored at −80°C until used.

Inhibitors—The inhibitors stock solutions were prepared as follows: CHI (1 mM), an inhibitor of protein synthesis, cantharidin (200 μM), an inhibitor of types 1 and 2A protein phosphatases (Li and Casida 1992, Li et al. 1993, MacKintosh and MacKintosh 1994), 6-dimethylaminopurine (DMAP; 1 mM), an inhibitor of protein kinases (MacKintosh and MacKintosh 1994, Comolli et al. 1996, Tobias and Wilson 1997) and dibucaine (2-butoxy-*N*-(2-diethylaminoethyl)-cinchonamide), a potent, long acting local anesthetic reagent (1 mM) which has been shown to inhibit Ca^{2+}-dependent protein kinases (Kawahara et al. 1980, Kasai and Field 1982, Wrenn and Wooten 1984, Mizuno 1994) were prepared as previously described (Mathooko et al. 1998). Dilutions were prepared in distilled water. All inhibitors were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Application of inhibitors—Fruit segments (10 cm long) were cut from the central part of the fruit and immersed in solutions containing the various inhibitors or distilled water/buffer (controls), vacuum-infiltrated and treated with humidified air or a humidified gas mixture consisting of 80% CO_2 and 20% O_2 as previously described (Mathooko et al. 1998).

Measurement of ethylene production—Fruit samples were enclosed in 1.5-liter plastic containers fitted with silicon rubber 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 head-space 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 of total RNA—Total RNA was isolated from the frozen samples according to the phenol-sodium dodecyl sulfate (SDS) method as described by Sambrook et al. (1989) with minor modifications.

Isolation of poly(A)$^+$ RNA—Poly(A)$^+$ RNAs were isolated by affinity chromatography using Oligo dT-30 (Takara, Kyoto, Japan) according to the manufacturer's protocol.

Plasmid DNA and radiolabeling—The cDNA fragments used in this study were cloned from cucumber fruit using reverse

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transcriptase-polymerase chain reaction (RT-PCR) and their full sequences were determined by rapid amplification of cDNA ends polymerase chain reaction (Shiomi et al. 1998). The actin cDNA (0.4 kb) used as an internal standard was cloned by RT-PCR using degenerated oligonucleotide primers (5'-GAR AAR ATG ACN CAR ATH ATG TT-3' as the upstream primer and 5'-ATR TCN ACR TCR CAY TTC AT-3' as the downstream primer). The primers were synthesized with reference to the conserved amino acid sequence of actin reported for other plant species. All inserts were isolated by digestion with the appropriate restriction enzymes followed by electrophoresis on 1% agarose gel in 40 mM Tris-acetate (pH 8.0) buffer containing 1 mM EDTA. After staining in ethidium bromide, the inserts were excised from the gel and purified by the Ultra Clean™ 15 DNA purification kit (Mo Bio Laboratories Inc., Solana Valley, CA, U.S.A.) following the manufacturer's instructions. The purified DNAs (30 ng) were labeled with [α-^{32}P]dCTP by the random primed DNA labeling kit (Boehringer, Mannheim, Germany) which labels DNA using random oligonucleotides as primers (Feinberg and Vogelstein 1983).

RNA blotting and northern hybridization analysis—Poly(A)$^+$ RNAs (3.2 μg per lane) were subjected to electrophoresis on a 1.2% agarose gel containing 0.66 M formaldehyde and 1×MOPS (20 mM MOPS, 5 mM sodium acetate and 1 mM EDTA) and were blotted overnight by capillary transfer to Hybond N nylon membranes (Amersham International) in 20× SSPE [1×SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA (pH 7.4)] according to the manufacturer's instructions. After transfer, the membranes were baked at 80°C for 30 min and the RNAs were cross-linked to the membranes by UV irradiation using a UV cross-linker (Amersham International). The membranes were prehybridized at 42°C for 2 h in a 10 ml solution containing 50% formamide (v/v), 5×Denhardt's reagent (1×Denhardt's reagent is 0.02% each of polyvinylpolypyrrolidone, Ficoll-400 and bovine serum albumin), 0.1% SDS, 5× SSPE, and 100 μg ml^{-1} of denatured fragmented herring sperm DNA. Hybridization was performed overnight in the same buffer solution containing 1.8–2.0×10^6 cpm denatured ^{32}P-labeled cDNA probes per ml against *CS-ACS1, CS-ACS2* and *CS-ACS3*. Following hybridization the membranes were washed once for 1 h at 60°C in 2×SSPE and 0.1% SDS and once at 60°C for 30 min each in 0.5× and 0.2×SSPE and 0.1% SDS. The membranes were subsequently exposed overnight to an imaging plate (Fuji Photo Film, Tokyo) at room temperature and the intensity of the hybridization signals was quantified using a BAS 2000 image analyzer (Fuji Photo Film, Tokyo). Equal reactivity and loading of poly(A)$^+$ RNA were confirmed by rehybridization with an actin cDNA after stripping off the former probes by washing the membranes in 0.1% SDS solution at 95°C for 5 min.

Results

Identification of CO_2 stress-inducible ACC synthase gene(s)—We previously demonstrated that the stimulation of ethylene biosynthesis in cucumber fruit under CO_2 stress is due to induction of the activities of both ACC synthase and ACC oxidase (Mathooko et al. 1998). In order to gather more information on the molecular basis for this induction and identify the CO_2 stress-inducible ACC synthase gene(s), we studied the expression pattern of three ACC synthase genes isolated from cucumber fruit, namely, *CS-ACS1, CS-ACS2* and *CS-ACS3*. Fruits were treated at 25°C for 24 h with air or elevated CO_2. In the control fruits ethylene production remained low while CO_2 stimulated ethylene production greatly (Fig. 1A). Northern blot analysis using poly(A)$^+$ RNAs showed that the stimulation of ethylene production by CO_2 paralleled the accumulation of only *CS-ACS1* transcript (Fig. 1B). No signals for *CS-ACS2* and *CS-ACS3* were detected. This indicates that *CS-ACS2* and *CS-ACS3* are not inducible by CO_2, at least, after 24 h treatment. However, these genes may be induced earlier in the treatment; to clarify this we

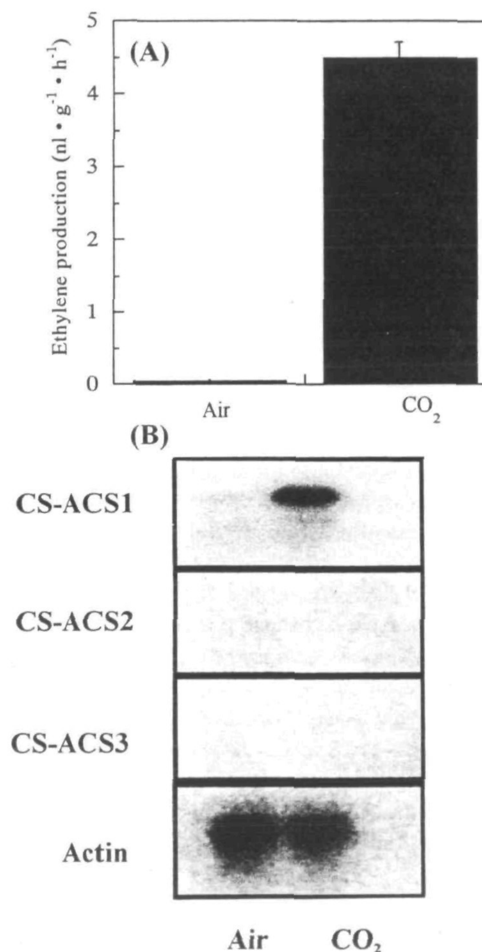

Fig. 1 Induction of ethylene production (A) and expression of *CS-ACS1, CS-ACS2* and *CS-ACS3* genes (B) in cucumber fruits under CO_2 stress. Fruits were treated for 24 h at 25°C in a continuous flow-through gas system with humidified air (control) or a gas mixture consisting of 80% CO_2 and 20% O_2. Ethylene production rate was determined after which samples were prepared for RNA extraction. Poly(A)$^+$ RNAs (3.2 μg per lane) were separated by electrophoresis on 1.2% formaldehyde-agarose gel, subjected to blotting on Hybond N nylon membranes and hybridized with equal amounts of ^{32}P-labeled gene-specific probes corresponding to the respective genes. The same membranes were stripped of the former probes and rehybridized with a probe for actin gene to normalize the amounts of poly(A)$^+$ RNAs loaded. The vertical bars are mean±SE of three replications.

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carried out time course studies.

Induction kinetics of the ACC synthase genes—Ethylene production in the control fruits remained at the basal level of 0.05–0.08 nl per gram per h (data not shown). The induction of ethylene production by CO_2 increased with time after a lag period of 6 h (Fig. 2A). Upon transfer of fruits to air for 12 h, ethylene production decreased to almost the control level. The accumulation of *CS-ACS1* transcript started 12 h after incubation in CO_2 and its time course was similar to that of ethylene production (Fig. 2B). Weak signals for *CS-ACS2* were detected 24 h after incubation in CO_2 and also upon transfer of fruits from CO_2 to air. No signal for *CS-ACS3* was detected throughout.

Effects of cycloheximide—For any induction of mRNA to qualify as a primary response, it should be insensitive to inhibitors of protein synthesis (Theologis et al. 1985). To investigate whether the expression of the ACC synthase genes studied here is a primary response to CO_2 stress, we vacuum-infiltrated fruit segments with varying concentrations of CHI, prior to treating with air or elevated CO_2. In the control fruits, we observed that although there was no change in ethylene production rate beyond the basal level, there was an increase in the accumulation of both *CS-ACS1* and *CS-ACS2* transcripts with increasing CHI concentration (data not shown). In fruit

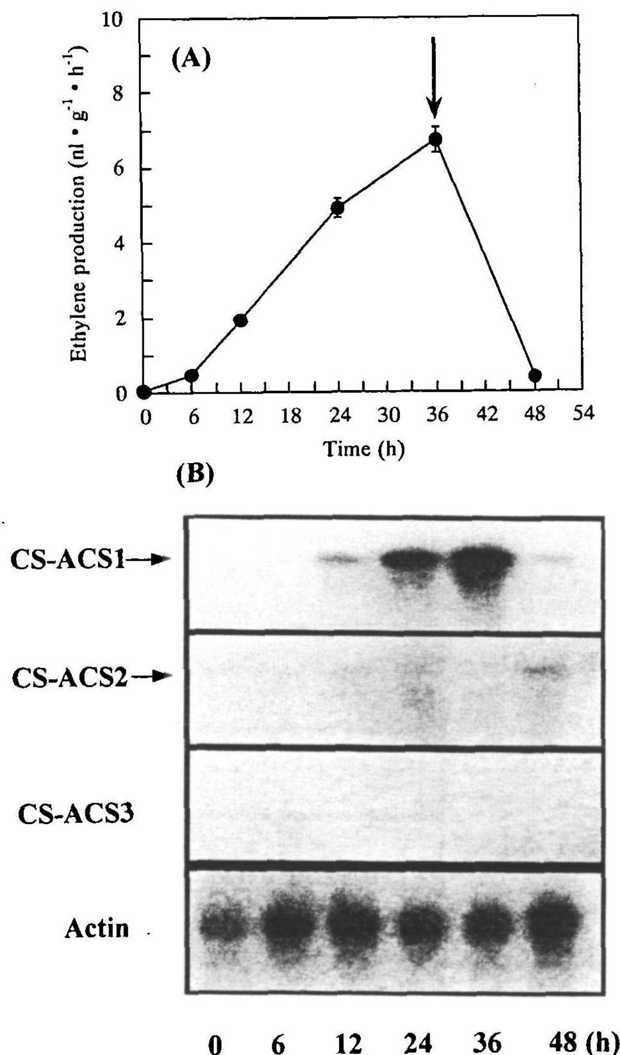

Fig. 2 Induction kinetics of ethylene production (A) and the expression of *CS-ACS1*, *CS-ACS2* and *CS-ACS3* genes (B) in cucumber fruits under CO_2 stress. Fruits were treated and poly(A)$^+$ RNAs (3.2 μg per lane) were analyzed by northern blotting as described in the legends of Fig. 1. The arrow indicates the time when fruits were transferred from CO_2-enriched atmosphere to air. The vertical bars are mean ± SE of three replications; where absent, the SE bars fall within the dimensions of the symbol.

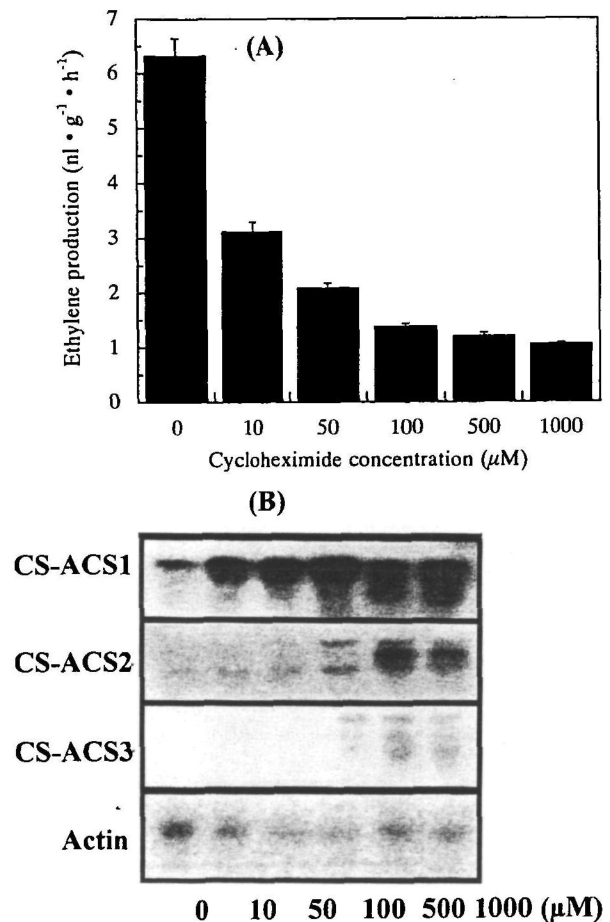

Fig. 3 Effects of various concentrations of cycloheximide (CHI) on ethylene production (A) and accumulation of *CS-ACS1*, *CS-ACS2* and *CS-ACS3* transcripts (B) in cucumber fruits under CO_2 stress. Fruit segments (10 cm long) vacuum-infiltrated with distilled water (control) or solutions containing the various concentrations of CHI were treated for 24 h at 25°C and poly(A)$^+$ RNAs (3.2 μg per lane) were analyzed by northern blotting as described in the legends of Fig. 1. The vertical bars are mean ± SE of three replications.

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segments treated concomitantly with CHI and CO_2, CHI inhibited CO_2 stress-induced ethylene production in a concentration-dependent manner (Fig. 3A). In contrast, there was superinduction of *CS-ACS1* transcript accumulation (Fig. 3B). For *CS-ACS2* and *CS-ACS3* weak signals were detected at 500 μM and 1,000 μM CHI.

Induction kinetics of the ACC synthase genes by cycloheximide—In order to obtain more information on the superinduction of the ACC synthase genes by CHI in cucumber fruit under CO_2 stress, fruits were infiltrated with 200 μM CHI prior to treatment with CO_2 for 36 h. At the indicated time points ethylene production rate was determined and samples prepared for RNA extraction. As previously reported (Mathooko et al. 1995a, 1998), in the presence of CHI induction of ethylene production by CO_2 decreased to about 10% and upon withdrawal of CO_2 ethylene production further declined to the control level (Fig. 4A). The accumulation of *CS-ACS1* transcript induced by CO_2 started after 6 h in the presence of CHI (Fig. 4B), while in the absence of CHI it started after 12 h (cf. Fig. 2). In contrast, the *CS-ACS2* transcript accumulated between 1 and 3 h but disappeared after 6 h. The accumulation of *CS-ACS2* transcript between 1 and 3 h was not observed in the absence of CHI (data not shown). Contrary to ethylene production, upon withdrawal of CO_2 there was little change in *CS-ACS1* transcript level, while there was a great increase in *CS-ACS2* transcript level.

Effects of protein kinase and protein phosphatase inhibitors on the expression of the ACC synthase genes—We

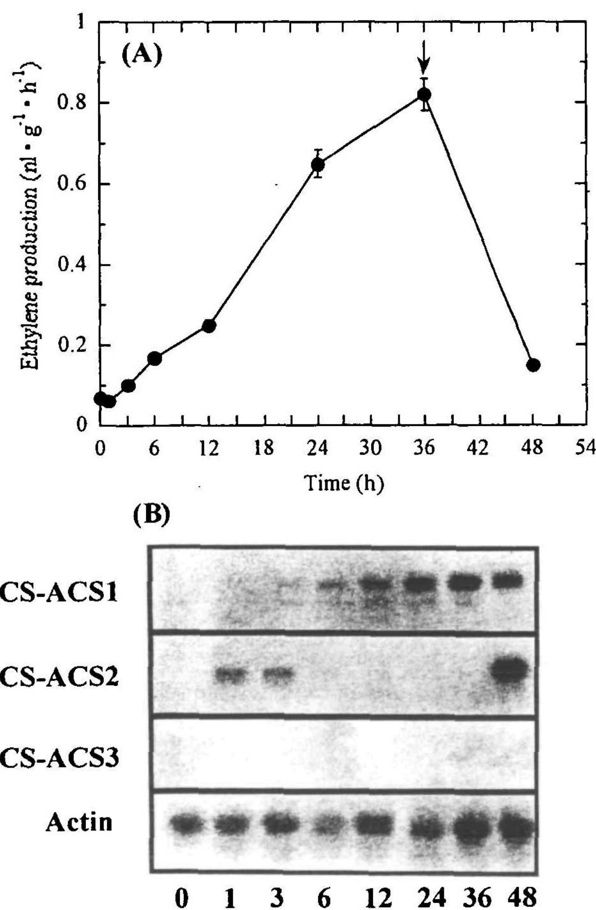

Fig. 4 Induction kinetics of ethylene production (A) and the expression of *CS-ACS1*, *CS-ACS2* and *CS-ACS3* genes (B) in CHI-pretreated cucumber fruits under CO_2 stress. Fruit segments (10 cm long) vacuum-infiltrated with 200 μM of CHI were treated and poly(A)$^+$ RNAs (3.2 μg per lane) were analyzed by northern blotting as described in the legends of Fig. 1. The arrow indicates the time when fruits were transferred from CO_2-enriched atmosphere to air. The vertical bars are mean ± SE of three replications; where absent, the SE bars fall within the dimensions of the symbol.

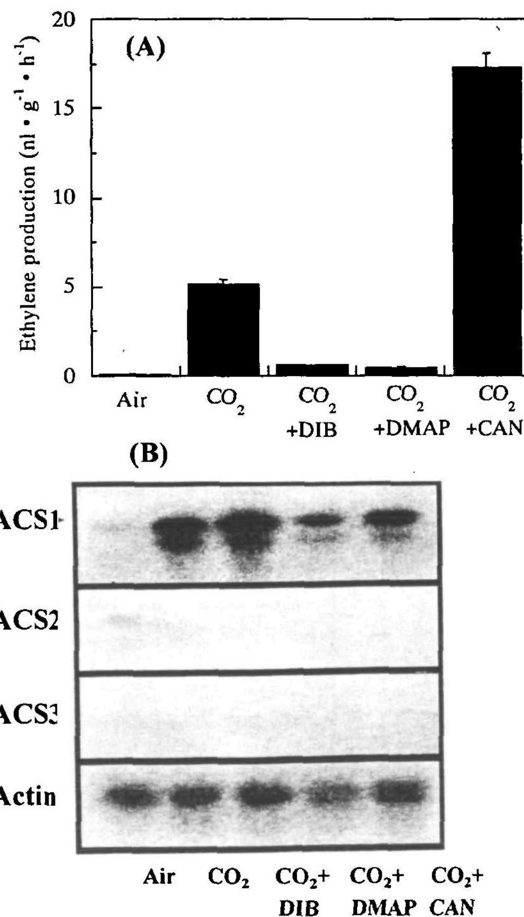

Fig. 5 Effects of protein kinase and protein phosphatase inhibitors on ethylene production (A) and the expression of *CS-ACS1*, *CS-ACS2* and *CS-ACS3* genes (B) in cucumber fruits under CO_2 stress. Fruit segments (10 cm) vacuum-infiltrated with distilled water (controls), 1 mM dibucaine (DIB), 1 mM 6-dimethylaminopurine (DMAP) or 100 μM cantharidin (CAN) were treated for 24 h and poly(A)$^+$ RNAs (3.2 μg per lane) were analyzed by northern blotting as described in the legends of Fig. 1. The vertical bars are mean ± SE of three replications.

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have previously demonstrated a possible involvement of protein phosphorylation and dephosphorylation in one or more of the steps in the CO_2 signal transduction pathway(s) that leads to induction of ethylene biosynthesis (Mathooko et al. 1998). In order to further understand the role of reversible protein phosphorylation in ACC synthase gene expression in cucumber fruits under CO_2 stress, fruits were treated with 1 mM dibucaine, 1 mM DMAP or 100 μM cantharidin prior to treatment with CO_2. Consistent with our previous observation (Mathooko et al. 1998), cantharidin stimulated CO_2 stress-induced ethylene production while dibucaine and DMAP inhibited it (Fig. 5A). DMAP seemed to slightly suppress the accumulation of *CS-ACS1* transcript below that caused by CO_2 alone but cantharidin and dibucaine had little effect (Fig. 5B).

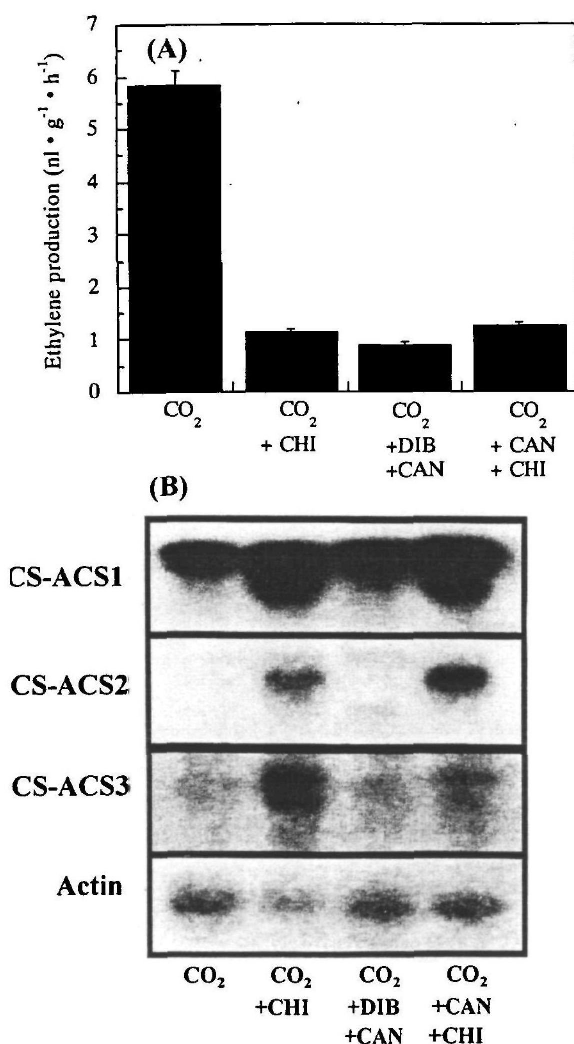

Fig. 6 Combined effects of cantharidin (CAN) and dibucaine (DIB) or cycloheximide (CHI) on ethylene production (A) and accumulation of *CS-ACS1*, *CS-ACS2* and *CS-ACS3* transcripts (B) in cucumber fruits under CO_2 stress. Fruit segments (10 cm long) vacuum-infiltrated with 0.2% dimethyl sulfoxide (control) or solutions containing 200 μM CHI, 100 μM CAN+1 mM DIB or 100 μM CAN+200 μM CHI were treated for 24 h and poly(A)$^+$ RNAs (3.2 μg per lane) were analyzed by northern blotting as described in the legends of Fig. 1. The vertical bars are mean ± SE of three replications.

To understand the interaction between inhibition of protein phosphatase and protein kinase and/or inhibition of protein synthesis, fruit segments were infiltrated with CHI, dibucaine and cantharidin, or cantharidin and CHI prior to treatment with CO_2. All these treatments inhibited CO_2 stress-induced ethylene production (Fig. 6A). A combination of CO_2 with CHI or with CHI and cantharidin superinduced at least genes for *CS-ACS1* and *CS-ACS2* (Fig. 6B). A combination of CO_2 with dibucaine and cantharidin had no effect on the accumulation of *CS-ACS1* transcript beyond that caused by CO_2 alone.

Discussion

The response of cucumber fruits to CO_2 stress with regard to induction of ethylene biosynthesis has well been characterized (Mathooko et al. 1995a, 1998). The stimulation of ethylene biosynthesis by various stimuli is due to induction of ACC synthase activity and subsequent accumulation of its transcript (Kende 1993). In the present study we have shown that similar to other stimuli, the members of the ACC synthase gene family in cucumber fruit are also differentially regulated under CO_2 stress. The *CS-ACS1* seems to be the main ACC synthase gene responsible for CO_2 stress-induced ACC synthase activity and ethylene production in cucumber fruits (Fig. 1A, 2A, Mathooko et al. 1995a, 1998). This gene, therefore, appears to be a common target for induction by both wounding and CO_2 stress. A similar link has been established in an anaerobically and auxin-regulated ACC synthase gene (Zarembinski and Theologis 1993) and the induction of *LE-ACC2* by wounding and pathogen infection in tomato fruit and leaf (Spanu et al. 1993). The decrease in the *CS-ACS1* transcript level upon withdrawal of CO_2 indicates that similar to ethylene production and ACC synthase activity (Mathooko et al. 1995a, 1998), CO_2 stress-induced accumulation of *CS-ACS1* transcript requires continuous presence of the gas, and further supports the role of this gene in CO_2 stress-induced ethylene biosynthesis. Continuous presence of CO_2 is also required for inhibition of ethylene biosynthesis (Kubo et al. 1990, Mathooko et al. 1995b, 1998) and ACC synthase gene expression (Mathooko et al. 1997, Rothan et al. 1997).

For any induction of mRNAs to qualify as a primary response, it must be insensitive to protein synthesis inhibition, indicating that the response to the stimuli is via modification of preexisting components (Ringhold 1979, Theologis et al. 1985, Herschman 1991). CHI induced the accumulation of *CS-ACS1* and *CS-ACS2* transcripts in the

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absence of CO_2. In the presence of CHI the induction of *CS-ACS1* transcript accumulation by CO_2 was relatively slow compared with that of *CS-ACS2* transcript (after 6 h; Fig. 4B) and continued for a long time, whereas that of *CS-ACS2* transcript was rapid (within 1 h; Fig. 4B), transient and accumulated to a higher level upon withdrawal of CO_2. Further, in the presence of CHI, the accumulation of *CS-ACS1* transcript did not disappear upon withdrawal of CO_2. It has been reported that inhibition of protein synthesis by CHI results in accumulation of some ACC synthase transcripts in the absence or presence of various stimuli, including auxin (Zarembinski and Theologis 1993, Abel et al. 1995, Kim et al. 1997, Yoon et al. 1997), wounding (Huang et al. 1991, Liang et al. 1992, Lincoln et al. 1993), Li^+ (Huang et al. 1991, Liang et al. 1996) and anaerobiosis (Zarembinski and Theologis 1993). On the other hand, CHI inhibited the expression of some ACC synthase genes (Kim et al. 1992, 1997) but it had no effect on the expression of some ACC synthase genes (Liu et al. 1993). These reports support the differential expression and regulation of the various members of the ACC synthase gene family. Although the mechanism(s) through which CHI induces gene expression is not known, it has been proposed that this could be due to: (a) transcriptional activation via prevention of synthesis or activation of a short-lived transcriptional repressor polypeptide whose subsequent physical or functional loss results in gene activation (Sen and Baltimore 1986, Mahadevan and Edwards 1991, Koshiba et al. 1995, Abel and Theologis 1996), (b) mRNA stabilization by a translational arrest-linked process or by preventing the synthesis of a labile nuclease (Huang et al. 1991, Liang et al. 1992, Koshiba et al. 1995, Liang et al. 1996), and (c) prevention of ribosome movement, with subsequent loading of additional ribosomes onto mRNA, thereby protecting the mRNA from RNase (Christopher and Good 1996).

Cantharidin stimulated while DMAP and dibucaine inhibited CO_2 stress-induced ethylene production, although these inhibitors had little effect on the accumulation of *CS-ACS1* transcript beyond that caused by CO_2 alone. We previously showed that cantharidin stimulated CO_2 stress-induced ethylene production, ACC synthase activity, and ACC accumulation while DMAP and dibucaine inhibited these (Mathooko et al. 1998). It is plausible, therefore, that the regulation of CO_2 stress-induced ethylene biosynthesis by reversible protein phosphorylation is not through enhanced accumulation of ACC synthase transcript but through other yet unknown mechanisms. In most cases, increased ethylene production rates appear to be correlated with enhanced ACC synthase transcription (Kende 1993, Fluhr and Mattoo 1996). However, transcription of ACC synthase genes may not be the only factor regulating the production of ACC; regulatory mechanisms at the posttranscriptional level may be equally important. These could be at the level of mRNA splicing, the control of translation or by modification of the native ACC synthase protein, including C- or N-terminal processing and covalent modification such as phosphorylation or alkylation. It is probable that cantharidin may 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 (Rottmann et al. 1991, Li and Mattoo 1994) without necessarily influencing the expression of the respective gene(s). To various degrees, there is experimental evidence that these posttranscriptional mechanisms of control apply to ACC synthase induced under stress conditions (Spanu et al. 1994, Olson et al. 1995, Zarembinski and Theologis 1997). Oetiker et al. (1997) showed that the expression kinetics of tomato ACC synthase genes *LE-ACS1B*, *LE-ACS3* and *LE-ACS4* were not correlated with elicitor-induced ethylene biosynthesis. When the accumulation of ACC synthase transcripts is not correlated with ethylene production and/or ACC synthase activity, then these observations may indicate that ethylene synthesis is under posttranscriptional control rather that transcriptional control of ACC synthase (Spanu et al. 1993, Oetiker et al. 1997).

In summary the increases in ACC synthase activity and ACC accumulation observed in cucumber fruit under CO_2 stress (Mathooko et al. 1998) which may lead to increased ethylene production are, at least in part, the result of accumulation of the transcripts for the *CS-ACS1* gene although other ACC synthase genes may be involved. The *CS-ACS1* gene may be also a primary response gene since it is insensitive to protein synthesis inhibition.

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