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Ethylene-Regulated Expression of ACC Oxidase and ACC Synthase Genes in Mung Bean Hypocotyls

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Ethylene induces an increase in transcript levels of the ACC oxidase gene (*VR-ACO1*) but suppresses expression of the ACC synthase gene (*VR-ACS1*) in etiolated mung bean hypocotyls. Here, treatment with either the transcription inhibitor α-amanitin or the protein synthesis inhibitor cycloheximide (CHI) completely abolished ethylene-induced accumulation of *VR-ACO1* mRNA. This indicated that ethylene-induction of *VR-ACO1* is due to transcriptional activation, which also relies on *de novo* protein synthesis. In contrast, CHI induced the accumulation of *VR-ACS1* transcripts. ABA also inhibited ethylene-induced *VR-ACO1* expression, but restored ethylene-suppressed *VR-ACS1* expression. Results of time-course experiments and an interaction analysis of CHI and ABA suggested that the latter may exert its effect by preventing the synthesis of a factor(s) necessary for ethylene action. Ethylene-signaling was studied in more detail, using two pharmacological inhibitors -- EGTA and sodium orthovanadate. Those experiments demonstrated that calcium ions and a Tyr type of protein phosphatase may be involved in regulating ethylene biosynthetic genes.

Keywords: ABA, ACC oxidase, ACC synthase, calcium, ethylene, mung bean

The gaseous plant hormone ethylene regulates a variety of physiological responses, and induces specific changes in gene expression during growth and development (Abeles et al., 1992; Kende, 1993; Kim et al., 2004). In higher plants, ethylene is synthesized via the following pathway: methionine → *S*-adenosyl-*L*-methionine (AdoMet) → 1 - aminocyclopropane-1-carboxylate (ACC) → ethylene. AdoMet is converted to ACC by ACC synthase, and the conversion of ACC to ethylene is catalyzed by ACC oxidase (Yang and Hoffman, 1984).

Ethylene is synthesized in response to various external stimuli, such as wounding, drought, anaerobiosis, pathogen attack, and auxin treatment (Yang and Hoffman, 1984). Ethylene production is also highly regulated through positive or negative feedback control on ACC synthase and/or ACC oxidase (Yang and Hoffman, 1984; Kende, 1993). The ability of exogenous or wound-induced ethylene to stimulate the activity of ACC oxidases has been observed in wounded cantaloupe and excised winter squash (Hoffman and Yang, 1982; Hyodo et al., 1993). Exogenous ethylene also markedly increases the level of ACC oxidase mRNA and its enzyme activities in mung bean and pea seedlings (Kim and Yang, 1994; Peck and Kende, 1995; Jin et al., 1999). Negative feedback control of ethylene production through the inhibition of ACC synthase activities has been recognized in a variety of fruits and vegetative tissues, including citrus fruits and mung bean seedlings (Riov and Yang, 1982; Yoshii and Imaseki, 1982).

Ethylene stimulates the expression of *VR-ACO1*, an ACC oxidase gene, and suppresses that of *VR-ACS1*, an ACC synthase gene, through positive and negative feedback mechanisms, respectively, in etiolated mung bean hypocotyls (Kim et al., 1997, 2001). Both phosphorylation and dephosphorylation are necessary for the ethylene-signaling that regulates the expression of those two genes (Kim et al., 1997). Jung et al. (2000) have suggested that the phosphoinositol-derived second messengers and cytosolic calcium are required for the ethylene-regulated expression of *VR-ACO1* in the root tissues of etiolated mung bean seedlings.

In addition to ethylene, other plant hormones, such as abscisic acid (ABA), auxins, cytokinins, and brassinosteroids, are involved in regulating ethylene production and biosynthetic genes in mung bean seedlings (Kondo et al., 1975; Kim et al., 1997, 2001; Yoon et al., 1997; Yi et al., 1999). Auxin is a well-known primary inducer, synergistically interacting with cytokinin or brassinosteroid to enhance ethylene production and expression of ACC synthase genes (Yi et al., 1999). In contrast, the effect of ABA appears to be complex, because that hormone, when applied with auxin, can inhibit auxin-induced ethylene production while low concentrations of ABA can slightly stimulate it (Kondo et al., 1975). Therefore, it remains unknown how ABA affects ethylene production.

Here, general pharmacological reagents were tested for their effects on the processes of gene expression and calcium homeostasis, as well as the status of dephosphorylation in the cell. Furthermore, a possible mechanism was investigated by which ABA might affect ethylene-regulated expression of *VR-ACO1* and *VR-ACS1* with respect to *de novo* protein synthesis and ethylene production.

MATERIALS AND METHODS

Plant Material and Incubation Conditions

Dry seeds of mung bean (*Vigna radiata* L.) were soaked overnight in aerated tap water. Seedlings were grown on 0.5% agarose plates for 3 d in the darkroom at 27°C. Batches (~0.5 g FW) of 0.5-cm hypocotyl segments were excised from a region 3 cm below the hook, and were transferred to scintillation vials (fitted with silicon plugs) that contained 0.5 mL of a medium comprising 200 μM AOA, 2% (w/v) sucrose, and 50 mM MES buffer (pH 6.2). The vials were incubated at 27°C under darkness for the indicated time periods. For some of the experiments, the media contained various types of supplements, including α-amanitin, cycloheximide, ABA, mannitol, EGTA, $LaCl_3$, or sodium ortho-

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vanadate in the presence or absence of 50 μL L^{-1} ethylene. All the reagents were purchased from Sigma-Aldrich (USA). At the end of the incubation periods, the tissues were either used to determine ethylene production, or the hypocotyl segments were assayed for *in vivo* ACC oxidase activity. They were collected, immediately frozen in liquid nitrogen, and stored at -80°C.

RNA Isolation and Northern Blot Analysis

Total RNA from mung bean hypocotyl segments was isolated as described previously (Kim et al., 1997). Total RNA (20 μg) was fractionated in a 1% formaldehyde-agarose gel and blotted to a nylon membrane (Amersham, USA). Equal loading of RNA was confirmed by viewing the gel under UV light at the end of the electrophoresis. The nylon membrane was hybridized to a ^{32}P-labeled *VR-ACO1* probe for ACC oxidase at 52°C (Kim et al., 1997). Blots were washed twice with a solution of 0.2X SSPE and 0.1% SDS at 65°C, and were autoradiographed at -80°C using Kodak XAR-5 film and an intensifying screen (DuPont, USA). For ACC synthase, the nylon membrane was boiled briefly in a 0.5% SDS solution to remove the previous probes, then hybridized with a ^{32}P-labeled *VR-ACS1* probe at 42°C (Kim et al., 1997). All experiments were repeated at least twice.

Determination of Ethylene Production

A 1-mL gas sample was withdrawn by syringe from the flask, and its ethylene level was assayed on a GC equipped with an aluminum column and flame ionization detector (Shimazu, Japan).

Measurement of *in Vivo* ACC Oxidase Activity

To assay the activities of ACC oxidase *in vivo*, hypocotyl segments were transferred to 50-mL Erlenmeyer flasks at the end of the incubation period. They were thoroughly rinsed twice with distilled water, and left for 15 min at room temperature in 2 mL of the same medium with gentle shaking. The flasks were then capped with silicon plugs, through which ACC was added by syringe to a final concentration of 2 mM. *In vivo* ACC oxidase activity was defined as the amount of ethylene produced within 30 min at 27°C under darkness. A 1-mL gas sample was analyzed as described above.

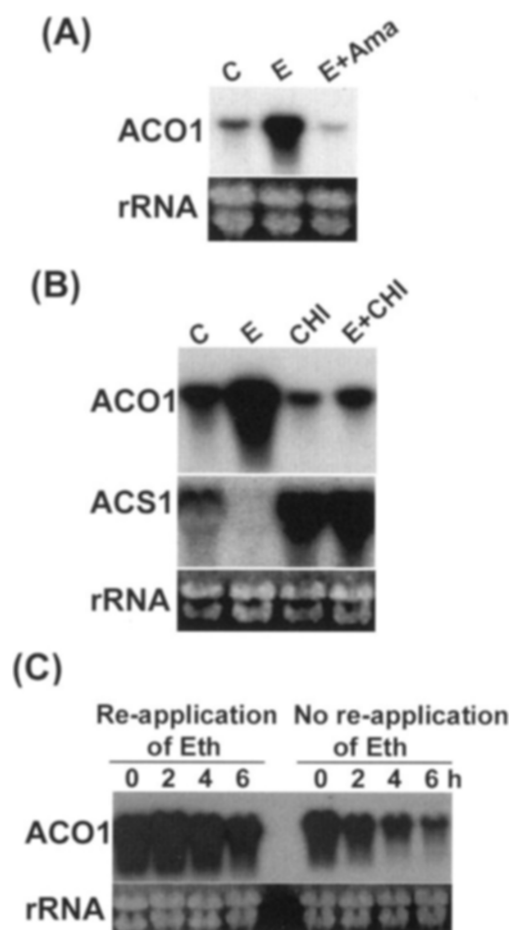

Figure 1. (**A**) Effect of α-amanitin on ethylene-induced accumulation of *VR-ACO1* mRNA. Hypocotyl segments were pre-incubated for 2 h in vials containing 0.5 mL medium, with or without 10 μM α-amanitin, then treated for 5 h with 50 μL L^{-1} ethylene. Afterward, total RNA was isolated and subjected to northern blot analysis. (**B**) Effect of CHI on transcript levels of *VR-ACO1* (upper panel) and *VR-ACS1* (lower panel). Hypocotyl segments were treated for 9 h with ethylene or 100 μM cycloheximide. (**C**) Determination of stability of *VR-ACO1* mRNA. Hypocotyl segments were treated with ethylene for 6 h, and then ethylene was removed by vacuum pump. Afterward, segments were treated with ethylene (left four lanes) or without ethylene (right four lanes) for times indicated. C, control; E and Eth, ethylene; Ama, α-amanitin; CHI, cycloheximide.

RESULTS

Effect of Transcriptional or Translational Inhibitors on Ethylene-induced Expression of the *VR-ACO1* Gene

In etiolated mung bean hypocotyls, ethylene induces the accumulation of ACC oxidase mRNA through a positive feedback mechanism (Kim et al., 1997). To evaluate whether the induction of *VR-ACO1* expression by ethylene occurs at the transcriptional or post-transcriptional level, hypocotyl segments were incubated for 2 h in the presence or absence of α-amanitin, an inhibitor of RNA polymerase II, before being treated with ethylene for 5 h. Total RNA isolated from those tissues was subject to northern blot analysis using *pVR-ACO1* as a probe for the ACC oxidase gene. The ethylene-induced accumulation of *VR-ACO1* transcripts was completely abolished by 10 μM α-amanitin (Fig. 1A), indicating that ethylene-induction of *VR-ACO1* mRNA was due to transcriptional activation.

Cycloheximide (CHI), an inhibitor of protein synthesis, completely inhibited the ethylene-induced accumulation of *VR-ACO1* transcripts (Fig. 1B, upper panel), indicating that *de novo* protein synthesis was required for ethylene action that causes the transcriptional activation of *VR-ACO1*. Expression of *VR-ACS1*, one of the ACC synthase genes, has been shown to be negatively controlled by ethylene (Kim et al., 1997) but is strongly induced by CHI in mung bean hypocotyls (Yoon et al., 1997). In the current study, the level of *VR-ACS1* mRNA declined in response to exogenous ethylene but was markedly increased by CHI alone (Fig. 1B, lower panel), observations consistent with previously reported results (Yoon et al., 1997). When applied together with ethylene, CHI completely blocked the ethylene-suppression of *VR-ACS1* expression, indicating that *de novo* protein synthesis also was required for ethylene action leading to the suppression of *VR-ACS1* expression.

Estimating the Stability of *VR-ACO1* mRNA

The stability of transcriptionally induced *VR-ACO1* mRNA

is determined by measuring the residual level of *VR-ACO1* mRNA at various time points after the removal of ethylene. Here, hypocotyl segments were treated with ethylene for 6 h to allow for near-maximal accumulation of *VR-ACO1* mRNA (Kim et al., 1997). Afterward, the segments were put under negative pressure for 5 min to remove gaseous ethylene. One group of segments was then treated again with ethylene while the other group remained untreated. Hypocotyl tissues that had been continuously supplied with ethylene displayed a maximal or near-maximal level of *VR-ACO1* mRNA for 6 h after the re-application of ethylene (Fig. 1C, left four lanes). However, those tissues not subjected to re-treatment showed a gradual, time-dependent, decrease in *VR-ACO1* mRNA, i.e., transcripts levels started to decline significantly at 2 h, diminishing to an amount less than half of the control at 4 h, with a further decrease thereafter (Fig. 1C, right four lanes). This result suggests that a conservative estimate of the half life of *VR-ACO1* mRNA should be less than 4 h.

Effect of ABA on *VR-ACO1* and *VR-ACS1* Expression

To investigate the effect of ABA on ethylene action in regulating the expression of *VR-ACO1* and *VR-ACS1*, hypocotyl segments were incubated for 9 h with ethylene or 100 μM ABA. Treatment with ABA alone substantially reduced the endogenous level of *VR-ACO1* mRNA and markedly increased that of *VR-ACS1* mRNA (Fig. 2A). When applied together with ethylene, ABA greatly decreased the ethylene-induced amount of *VR-ACO1* transcripts to less than that of the control, but increased the ethylene-suppressed level of *VR-ACS1* mRNA (Fig. 2A). These results demonstrate that ABA inhibited the ethylene action involved in the regulation of both *VR-ACO1* and *VR-ACS1* expression.

ABA apparently antagonizes the effect of endogenous ethylene that might be responsible for the low levels of *VR-ACO1* and *VR-ACS1* mRNA. This may be inferred because the control level of *VR-ACO1* mRNA declined while that of *VR-ACS1* increased in response to ABA alone (Fig. 2A, lane 3). Therefore, it is possible that endogenous ABA may be involved in regulating the basal level of *VR-ACO1* and *VR-ACS1* expression. To test for any physiological significance of this ABA effect, hypocotyl segments were incubated in media with or without 0.5 M mannitol, in the presence of ethylene. Mannitol is well known for inducing osmotic stress, which usually results in an increase in the endogenous level of ABA, thereby regulating the expression of ABA-responsive genes (Williams et al., 1994; Lehmann et al., 1995). Here, mannitol caused a significant decrease in the ethylene-induced level of *VR-ACO1* mRNA and an increase in the ethylene-suppressed level of *VR-ACS1* mRNA (Fig. 2B). This result is consistent with the notion that a change in the level of endogenous ABA may participate in regulating the expression of ethylene biosynthetic genes, probably contributing to final ethylene production.

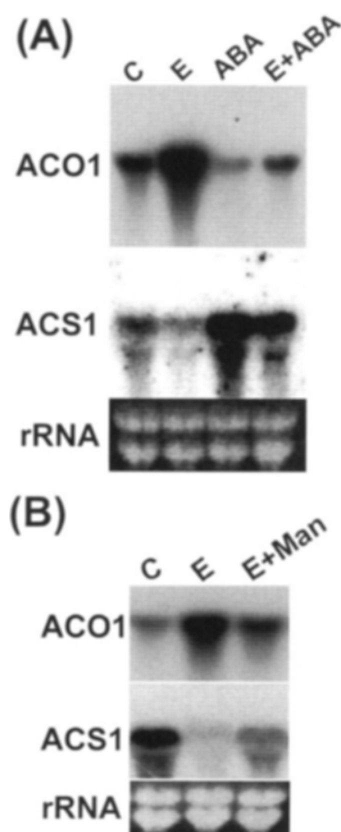

Figure 2. (**A**) Effect of ethylene and ABA on transcript levels of *VR-ACO1* (upper panel) and *VR-ACS1* (lower panel). Where indicated, hypocotyl segments were treated for 9 h with ethylene or 100 μM ABA. (**B**) Effect of mannitol on transcript levels of *VR-ACO1* (upper panel) and *VR-ACS1* (lower panel). Where indicated, hypocotyl segments were treated for 8 h with 0.5 M mannitol (Man). C, control; E, ethylene.

Correlation between the Effects of CHI and ABA on *VR-ACO1* and *VR-ACS1* Expression

As mentioned above, CHI and ABA exerted apparently similar effects on *VR-ACO1* and *VR-ACS1* expression with respect to their strength and mode of action. To further compare these influences, time-course experiments were conducted to monitor changes in the transcript levels of *VR-ACO1* and *VR-ACS1* in the presence of ethylene, 100 μM CHI, or 100 μM ABA. The amount of *VR-ACO1* transcripts increased substantially 2 h after ethylene treatment, reaching a maximum detectable level at 8 h (Fig. 3A, upper panel). However, in the presence of CHI or ABA, the ethylene-induced accumulation of *VR-ACO1* transcripts was not observed at 2 h, but, rather, their amounts tended to decline over the incubation period. By contrast, *VR-ACS1* transcripts disappeared at 2 h and remained undetectable thereafter (Fig. 3A, lower panel). Treatment with both ethylene and CHI, or both ethylene and ABA, resulted in *VR-ACS1* mRNA transcripts increasing to near maximal levels within 1 h after incubation. Altogether, these results demonstrate that CHI and ABA may share a common time-dependent mode of action with regard to both *VR-ACO1* and *VR-ACS1* expression.

The ethylene-induced accumulation of *VR-ACO1* mRNA was completely inhibited when hypocotyl segments were treated with ABA or CHI alone, but was not further hindered when a combination of both was applied (Fig. 3B, upper panel). Similarly, no further stimulating effect on *VR-ACS1* expression was observed with both ABA and CHI, although, individually, they strongly stimulated the accumulation of *VR-ACS1* mRNA (Fig. 3B, lower panel). When these results are considered along with those from the time-course experiments, one can conclude that the absence of any

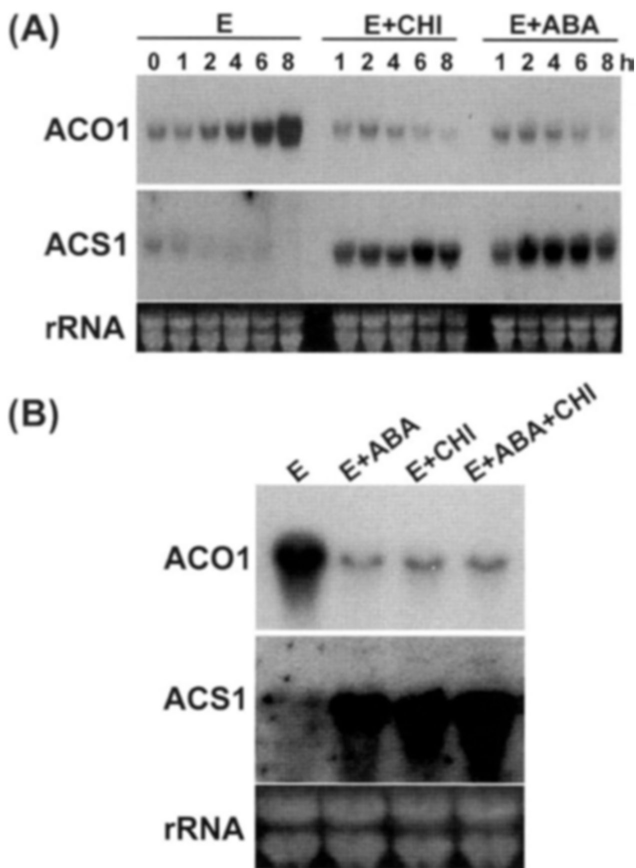

Figure 3. (**A**) Time course of CHI and ABA effects on ethylene-induced levels of *VR-ACO1* transcripts (upper panel) and ethylene-suppressed *VR-ACS1* (lower panel). Hypocotyl segments were treated with ethylene (E), 100 μM CHI, or 100 μM ABA for times indicated. (**B**) Interaction analysis of ABA and CHI effects on transcript levels of *VR-ACO1* (upper panel) and *VR-ACS1* (lower panel). Hypocotyl segments were treated for 9 h with ethylene, 100 μM CHI, or 100 μM ABA.

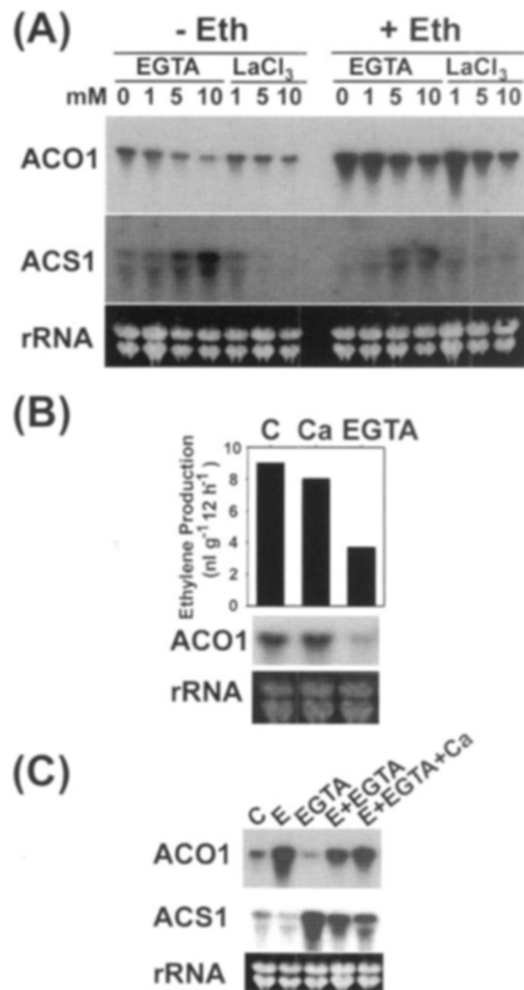

Figure 4. (**A**) Effect of EGTA and $LaCl_3$ on transcript levels of *VR-ACO1* (upper panel) and *VR-ACS1* (lower panel). Hypocotyl segments were treated for 9 h with various concentrations of EGTA or $LaCl_3$. (**B**) Effect of $CaCl_2$ and EGTA on ethylene production. Hypocotyl segments were treated with 1 mM $CaCl_2$ or 10 mM EGTA either for 12 h to determine ethylene production, or 8 h for northern blot analysis. (**C**) Reversibility of EGTA effect by addition of $CaCl_2$. Hypocotyl segments were treated for 9 h with 10 mM EGTA or 10 mM $CaCl_2$. C, control; E, ethylene.

additive effect between CHI and ABA raises the possibility that they may be involved in the same pathway leading to the inhibition of ethylene action.

Possible Involvement of Calcium Ions in the Regulation of Ethylene Biosynthetic Genes

Cytosolic calcium functions as a second messenger in a number of signal transduction pathways in plant cells (for a review, see Bush, 1995). Therefore, an experiment was designed to test whether Ca might participate in ethylene-signaling for the induction of *VR-ACO1* and the suppression of *VR-ACS1* expression. Mung bean hypocotyl tissues were incubated with various concentrations of EGTA or $LaCl_3$ in the absence or presence of ethylene. EGTA, a calcium chelator, substantially reduced the amount of *VR-ACO1* transcripts in the absence of ethylene, whereas it stimulated *VR-ACS1* expression (Fig. 4A, left lanes). $LaCl_3$, a plasma membrane calcium channel blocker, reduced the levels of both *VR-ACO1* and *VR-ACS1* mRNA (Fig. 4A). This same pattern was also observed in the presence of ethylene, i.e., EGTA and $LaCl_3$ inhibited the ethylene-dependent induction of *VR-ACO1* expression whereas *VR-ACS1* expression was stimulated by EGTA but not by $LaCl_3$ (Fig. 4A, right lanes).

To determine the physiological relevance of calcium ions for ethylene production, hypocotyl tissues were incubated with 1 mM $CaCl_2$ or 10 mM EGTA. Afterward, the level of *VR-ACO1* transcript and the amount of ethylene produced were measured (Fig. 4B). EGTA reduced both ethylene and *VR-ACO1* mRNA more than two-fold compared with the control, although exogenous treatment with 1 mM $CaCl_2$ caused no significant change. This was probably because of the high concentration of extracellular calcium present in the cell-wall compartment (Fig. 4B). One possible explanation for this inhibition of ethylene production and *VR-ACO1* expression by EGTA was simply the toxic effect by this excessive inhibitor. To clarify this, a test was conducted to examine whether this EGTA inhibition could be reversed by the addition of 10 mM $CaCl_2$. In fact, this inhibition of *VR-ACO1* mRNA accumulation by EGTA was slightly recovered by the excessive concentration of $CaCl_2$ (Fig. 4C). Furthermore, the EGTA-stimulation of *VR-ACS1* expression was slightly inhibited by Ca treatment. Therefore, these results are indicative of the

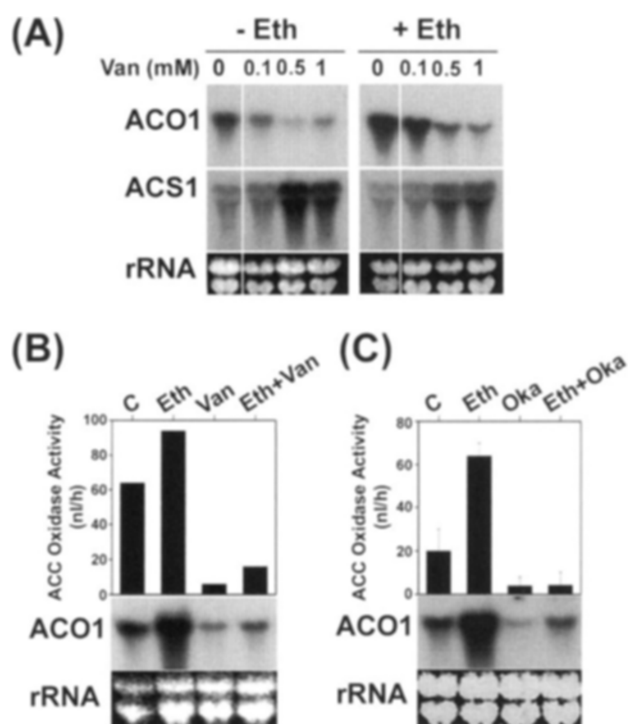

Figure 5. (**A**) Effect of sodium orthovanadate on transcript levels of *VR-ACO1* (upper panel) and *VR-ACS1* (lower panel). Hypocotyl segments were treated for 9 h with various concentrations of sodium orthovanadate (Van). (**B**) Effects of sodium orthovanadate on *in vivo* activity of ACC oxidase enzyme and the transcript level of *VR-ACO1*. Hypocotyl segments were treated for 9 h with 0.5 mM sodium orthovanadate, after which one group of segments was subject to *in vivo* assay of ACC oxidase enzyme, while the other group was used for isolating total RNA. (**C**) Effects of okadaic acid on *in vivo* activity of ACC oxidase and on transcript level of *VR-ACO1*. Hypocotyl segments were treated for 9 h with 1 μM okadaic acid (Oka). C, control; Eth, ethylene.

reversibility of the EGTA effect by calcium.

Possible Involvement of Protein Tyr Phosphatases in the Regulation of Ethylene Biosynthetic Genes

By employing pharmacological inhibitors that antagonize the Ser/Thr-type of protein kinases and protein phosphatases, it has been previously shown that protein phosphorylation and dephosphorylation events are required for ethylene-signaling involved in the regulation of *VR-ACO1* and *VR-ACS1* expression (Kim et al., 1997). Furthermore, the phosphorylation and dephosphorylation of Tyr residues of proteins may also serve as important a function in signal-transduction in plants as in animals (Luan, 1998; Kaneyama et al., 2000; Rayapureddi et al., 2005). Here, the Tyr type of protein dephosphorylation was studied for its potential involvement in the ethylene regulation of *VR-ACO1* and *VR-ACS1* expression using sodium orthovanadate, which is a well-known inhibitor of Tyr-specific protein phosphatases (Rayapureddi et al., 2005). In a dose-dependent manner, vanadate treatment reduced the ethylene-induced level of *VR-ACO1* mRNA as well as the endogenous level of *VR-ACO1* mRNA (Fig. 5A, upper panel). By contrast, vanadate strongly increased the level of *VR-ACS1* mRNA both in the presence and absence of ethylene.

To examine whether there was any physiological relevance for this change in the ethylene-induced *VR-ACO1* mRNA level in response to vanadate, ACC oxidase activities were determined. Hypocotyl segments were incubated in a combination of ethylene and vanadate for 9 h, after which they were assayed for *in vivo* activity. Enzyme levels were greatly decreased, reflecting the reduced amount of *VR-ACO1* transcripts (Fig. 5B). These results suggest that vanadate-sensitive protein dephosphorylation may play a role in ethylene-signaling, leading to the regulation of ACC oxidase activity. Okadaic acid, an antagonist of the Ser/Thr type of protein phosphatases, also inhibits *VR-ACO1* expression but stimulates that of *VR-ACS1* (Kim et al., 1997). Here, okadaic acid also strongly inhibited the ethylene-induction of *in vivo* ACC oxidase activity, with a concomitant decrease in the level of *VR-ACO1* transcripts (Fig. 5C).

DISCUSSION

The ethylene-regulated expression of *VR-ACO1* and *VR-ACS1* is inversely correlated through positive and negative feedback controls, respectively (Kim et al., 1997, 2001). That is, hormones, such as auxin and cytokinin, as well as pharmacological reagents, e.g., staurosporine and okadaic acid, which inhibit ethylene action, always result in a reverse pattern with respect to this regulation (Kim et al., 1997, 2001). In the current study, almost all of the tested reagents -- CHI, ABA, mannitol, EGTA, and vanadate -- also exerted inverse effects on gene expression. These results are highly consistent with the notion that expression of *VR-ACO1* and *VR-ACS1* is tightly coupled together in a common pathway of ethylene-signaling. The reason for the inverse coupling is unclear. It may be advantageous for a plant system to prevent hypocotyl tissues from overproducing ethylene in response to particular internal or external stimuli. This can be done by balancing the ACC content and its conversion to ethylene.

However, it should also be noted that other genes encoding ACC synthases in mung bean, such as *VR-ACS6* and *VR-ACS7*, are not necessarily regulated by the same inverse relationship as with the *VR-ACO1* gene (Yoon et al., 1997; Yi et al., 1999). CHI, an inhibitor of protein synthesis, induces *VR-ACS1* expression in mung bean hypocotyls (Kim et al., 1996; Yoon et al., 1997), suggesting that its inductive effect may either be due to the inhibited synthesis of a short-lived repressor or because of mRNA stabilization through the depletion of a labile nuclease(s) (Yoon et al., 1997). Here, CHI also increased the ethylene-suppressed level of *VR-ACS1* mRNA (Fig. 1B), while, in contrast, it completely inhibited the transcriptional activation of *VR-ACO1* (Fig. 1B). This indicates that ethylene-signaling requires *de novo* protein synthesis. Based on the tight and inverse coupling between *VR-ACO1* and *VR-ACS1* expressions described above, it is likely that the induction of *VR-ACS1* by CHI may have resulted from the inhibition of ethylene action suppressing that expression. This may also be true for ABA: in either the absence or presence of exogenous ethylene, ABA inhibited ethylene action involved in transcriptional activation of *VR-ACO1* but induced *VR-ACS1* expression (Fig. 2A).

Likewise, one can propose that CHI and ABA interfere with ethylene-signaling through a common pathway, because both components acted in the same time-dependent manner to inhibit ethylene-induced *VR-ACO1* expression while also inducing ethylene-suppressed *VR-ACS1* expression (Fig. 3A). The fact that no cooperative effect between CHI and ABA was detected (Fig. 3B) supports the notion that they may not act independently. Moreover, a

discrepancy existed in lag times between the inhibitory and stimulatory effects of CHI or ABA. Briefly, their inhibition of ethylene-induced *VR-ACO1* expression was manifested 2 h after treatment whereas their stimulation of *VR-ACS1* expression occurred within 1 h (Fig. 3A). It is apparently difficult to reconcile these differences if CHI and ABA interfere with the common pathway that simultaneously leads to inhibition and stimulation. However, when one considers that the half life of *VR-ACO1* mRNA is about 4 h (Fig. 1B), it is very likely that the inhibitory effect of CHI or ABA on ethylene-induction of *VR-ACO1* may have occurred much earlier than 2 h post-treatment. Although the precise regulatory mechanism for ABA remains to be determined, several researchers have demonstrated that it shares a commonality with CHI in regulating gene expression (Agrawal et al., 2003; Kim et al., 2003; Knight et al., 2004). For example, a promoter region of the CBF genes is responsible for CHI- or ABA-induced CBF expression (Knight et al., 2004).

When ABA and IAA are simultaneously applied to tissues, the former inhibits IAA-induced ethylene production whereas low concentrations slightly increase that production (Kondo et al., 1975). This apparent contradiction by ABA may be due to its reverse effect on the expression of ethylene biosynthetic genes, i.e., here, ABA induced expression of *VR-ACS1* whereas it suppressed ethylene-inducible expression of *VR-ACO1* (Fig. 3).

Cytosolic free Ca is a well-known second messenger in a variety of signal-transduction pathways in plant cells (Bush, 1995). Calcium ions act as second messengers in ethylene-induced chitinase and aerenchyma formation (Raz and Fluhr, 1992; He et al., 1996). Jung et al. (2000) also have demonstrated that those ions are involved in ethylene-induced activation of the ACC oxidase gene in mung bean roots. Consistent with those findings, the current study also showed that a calcium chelator, EGTA, significantly inhibited ethylene action, resulting in a reduced level of *VR-ACO1* mRNA and an increased level of *VR-ACS1* mRNA in the hypocotyls. Furthermore, ethylene production declined in response to EGTA application, correlated with the reduced level of *VR-ACO1* mRNA (Fig. 4). Jung et al. (2000) have reported that EGTA reduces *in vivo* activity of the ACC oxidase enzyme, accompanied by a corresponding decline in the amount of ACC oxidase protein in mung bean root tissues. Altogether, these results are compelling evidence that cytosolic calcium does act as a second messenger in ethylene-signaling, ultimately contributing to the regulation of ethylene production in a physiological context. Interestingly, $LaCl_3$, a calcium channel blocker of the plasma membrane, not only effectively reduced the level of *VR-ACO1* but also completely abolished *VR-ACS1* expression (Fig. 4A). This is very exceptional because the regulation of *VR-ACO1* and *VR-ACS1* has been always shown to be tightly coupled in an inverse manner. This particular response might have resulted from a general inhibition of mRNA synthesis due to the toxic effects of a high concentration of $LaCl_3$ because, unlike in the case with EGTA, the addition of excessive $CaCl_2$ failed to reverse the inhibitory effects of $LaCl_3$ on the expression of both ethylene biosynthetic genes (data not shown).

Ethylene-signaling includes a phosphorylation cascade, probably mediated by a MAPK kinase pathway (Chen et al., 2005). In animals, MAPKs are activated by dual phosphorylation on Thr and Tyr residues and, in turn, are inactivated by phosphatases with dual-specificity for both Ser/Thr and Tyr residues (Luan, 1998). Plant proteins also are phosphorylated on Tyr residues. For example, the *Arabidopsis* proteome contains a number of Tyr-specific protein kinases, as well as a Tyr-specific phosphatase (AtPTP1) and 17 dual-specificity phosphatases (Carpi et al., 2002). When studied *in vitro*, the phosphatase activity of AtPTP1 is inhibited by vanadate, a specific inhibitor of protein Tyr phosphatases, but not by okadaic acid, which is an inhibitor of the Ser/Thr type of protein phosphatases (Xu et al., 1998). Rayapureddi et al. (2005) have also reported another type of Tyr-specific phosphatase in *Arabidopsis*. In addition to those Tyr-specific phosphatases, a dual-specificity protein phosphatase has been cloned and characterized to inactivate a MAP kinase in *A. thaliana* (Gupta et al., 1998). In the present study, vanadate inhibited *VR-ACO1* expression with a concomitant reduction in the activity of the ACC oxidase enzyme and the activation of *VR-ACS1* expression, both under the control of ethylene. This suggests that the MAPK kinase pathway, involved in ethylene-signaling, may include an event of protein dephosphorylation from Tyr residues. In addition, ethylene-signaling here also required an event of protein dephosphorylation from Ser/Thr residues because okadaic acid completely inhibited *VR-ACO1* expression with a corresponding reduction in the level of ACC oxidase activity (Fig. 5C). The results of previous research, which used pharmacological reagents specific to Ser/Thr type of protein kinases and phosphatases, have led to the theory that the phosphorylation/dephosphorylation events required for ethylene-signaling are involved in regulating *VR-ACO1* and *VR-ACS1* expression in mung bean hypocotyl tissues (Kim et al., 1997).

Although the nature of the ABA receptor remains unknown, several signaling intermediates in ABA-regulated gene expression have been well studied (Leung and Giraudat, 1998). For example, ABA-induced accumulation of dehydrin mRNA is reduced by both K252a and okadaic acid, respective inhibitors of protein kinases and phosphatases (Hey et al., 1997). Molecular cloning of the *ABI1* and *ABI2* genes from *A. thaliana* has revealed that they encode protein phosphatase 2C (Leung et al., 1997). In addition, ABA has been shown to induce an increase in cytosolic Ca concentrations in the cells of corn coleoptiles and parsley hypocotyls and roots (Gehring et al., 1990). Calcium is also involved in ABA-signaling in vegetative tissues, including rice (Frandsen et al., 1996; Leung and Giraudat, 1998). Hwang and Lee (2001) have reported that an ABA response in the guard cells is mediated by changes in both the status of calcium and protein phosphorylation. Here, the regulation of *VR-ACO1* and *VR-ACS1* gene expression by ethylene was inversely coupled to a common pathway that required cytosolic Ca and protein dephosphorylation. Therefore, it is plausible that ABA may interfere with the calcium and phosphorylation/dephosphorylation status in mung bean hypocotyls, resulting in the regulation of ethylene biosynthetic genes.

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