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Sequential induction of the ethylene biosynthetic enzymes by indole-3-acetic acid in etiolated peas

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Abstract

Ethylene induced an increase in the accumulation of 1-aminocyclopropane-1-carboxylate (ACC) oxidase transcript level and enzyme activity in the first internode of 5- to 6-day-old etiolated pea (*Pisum sativum* L.) seedlings. Indole-3-acetic acid (IAA), which stimulates ethylene production by enhancing ACC synthase activity, also caused an increase in ACC oxidase transcript and activity levels. The IAA-induced increase in ACC oxidase mRNA level and enzyme activity was blocked by 2,5-norbornadiene (NBD), a competitive inhibitor of ethylene action. This indicates that IAA induced ACC oxidase through the action of ethylene. The level of ACC synthase mRNA and enzyme activity started to increase less than 1 h after the start of IAA treatment, whereas ACC oxidase activity and transcript levels began to rise after 2 h of IAA treatment. These results indicate that the enzymes of ethylene biosynthesis are sequentially induced after treatment of intact pea seedlings with IAA. The increase in ACC synthase activity leads to the production of ACC, which is converted by the low constitutive level of ACC oxidase activity to ethylene. Through a positive feedback loop, ethylene promotes the accumulation of ACC oxidase mRNA and the increase in ACC oxidase activity.

Introduction

Ethylene is a gaseous hormone that regulates a wide range of physiological responses in vegetative tissues [1]. In etiolated pea seedlings, an asymmetric distribution of ethylene biosynthesis may be involved in the asymmetry of growth that leads to the formation of the apical hook [19]. This asymmetry in ethylene production may result from a gradient in auxin concentration between the outer and inner portions of the apical hook. Ethylene production in vegetative tissues is thought to be regulated by the level of free IAA [24]. While it is difficult to establish the exact auxin concentration at its site of action, experiments with ^{3}H-IAA show that the IAA level within the interior portion of the hook is approximately 4-fold greater than in the outer portion of the hook [21]. Although auxin is normally associated with cell elongation, higher concentrations (3 μM to 1 mM) of IAA inhibit growth by stimulating ethylene production [3]. Thus, a high local concentration of IAA in the inner portion of the hook may be responsible for the increased ethyl-

ene production in this region [19], and this may, ultimately, lead to inhibition of growth. To understand how these two hormones may interact in this response, we are studying the effects of IAA on the enzymes of ethylene biosynthesis.

Ethylene production is normally low in vegetative tissues but can be increased by a variety of stimuli, including wounding and IAA [1]. In most cases, the increase in ethylene production appears to result from an increase in the transcript and activity levels of ACC synthase, the first committed enzyme of ethylene biosynthesis [9]. ACC synthase catalyzes the formation of ACC from *S*-adenosyl-L-methionine (AdoMet). ACC is converted to ethylene by the second enzyme, ACC oxidase, which is thought to be constitutively present in most vegetative tissues [24]. Because a stimulus that promotes ethylene production usually causes ACC synthase activity to increase rapidly from extremely low or undetectable levels, and because ACC oxidase is usually constitutively present at least at low levels, ACC synthase has been widely regarded as the rate-limiting step in ethylene biosynthesis [24]. There is evidence, however, that changes in ACC oxidase levels may also contribute to the overall increase in rates of ethylene production. Using *in vivo* assays, it has been shown that ethylene pretreatment increases ACC oxidase activity in citrus leaves [16], etiolated pea seedlings [20], and carnation petals [5], and wounding of ripening tomato fruit induces the accumulation of ACC oxidase transcript levels [6]. Wounding also causes an increase in ACC oxidase activity levels in winter squash mesocarp [7] and in ACC oxidase transcript and activity levels in etiolated mung bean hypocotyls [11]. In these cases, wound-induced accumulation of ACC oxidase could be blocked by treatment with NBD, a competitive inhibitor of ethylene action [22]. In orchid flowers, pollination caused an increase in ACC oxidase transcript levels, and this increase could be inhibited by NBD [13]. These results show that a stimulus causing an increase in ethylene production can also cause an increase in ACC oxidase activity. It also has been suggested that the enzymes of ethylene biosynthesis are sequentially induced by a stimulus promoting ethylene production [7]. It is currently not known whether, in these instances, ACC oxidase is a rate-limiting enzyme in the production of ethylene. In the present work, we report on the effects of IAA on ACC oxidase transcript and activity levels. We also compare the timing of the induction of both ACC synthase and ACC oxidase. For our studies, we are using the first internode of 5- to 6-day-old etiolated peas because this tissue has a very low basal level of ethylene production [20], making it easy to detect changes in ACC synthase and ACC oxidase mRNA and enzyme activity levels

Materials and methods

Plant material

Pea seeds (*Pisum sativum* L. cv. Alaska; Cliston Seed Co., Faison, NC) were imbibed overnight in aerated tap water. Seedlings were grown in vermiculite at 25 °C in darkness. In all experiments, treatments were performed on intact, 5- to 6-day-old seedlings that had formed a second node, and the start of the treatments was designated as 0 h. At the times indicated, 2 cm sections were isolated from the seedlings and immediately frozen in liquid nitrogen. Tissue from the same experiment was used for both the enzyme assays and RNA isolations. All manipulations were performed under safe green light (530–590 mm).

Hormone and inhibitor treatment of plant material

For ethylene treatments, seedlings were enclosed in 9 l desiccators fitted with injector ports that were sealed with rubber serum vial caps. Ethylene was injected into the desiccators, and the proper internal ethylene concentration was confirmed by gas chromatography. Purafil (Purafil, Norcross, GA), which absorbs ethylene, was added to control treatments in a dish at the bottom of the desiccator to prevent accumulation of ethylene produced by the seedlings. IAA solutions contained 100 μM IAA, 0.05% ethanol, and 0.05%

Tween-20, adjusted to pH 6.0 with dilute NaOH. Control solutions of the same pH were identical except that IAA was omitted. To disturb the seedlings as little as possible, the solutions were applied to the intact plants as a fine mist using a spray bottle. Treatments with NBD were performed in 9 l desiccators. Immediately after being treated with IAA or control solutions, the seedlings were placed in the chamber and NBD was injected to yield a concentration of 3000 μl/l in the gas phase.

Measuring the rate of ethylene production

At the indicated times after treatments, 7 to 10 sections were isolated from the first internode and enclosed in 4 ml tubes fitted with serum vial caps. After 30 min, 1 ml of headspace was withdrawn for determination of ethylene concentration by gas chromotography. Because the lag time for wound-ethylene production in etiolated pea stems is ca. 26 min [17], taking the measurements after 30 min minimizes any contributions of wound effects to the ethylene production rates.

ACC oxidase enzyme assays

The ACC oxidase assay was modified from that of Ververidis and John [23]. For the experiments with IAA treatment, weighed, frozen stem sections were ground in liquid nitrogen. An extraction buffer (1 ml/g FW) consisting of 100 mM Tris-HCl pH 7.2, 30 mM sodium ascorbate, and 10% glycerol was added to the frozen powder which was allowed to thaw to a slurry. The total extract was transferred to a cold, 1.5 ml microcentrifuge tube on ice and centrifuged at 15000 $\times$ *g* for 10 min at 4 °C. The supernatant was removed to a fresh tube on ice and used directly in the assay. Neither polyvinylpolypyrrolidone nor Triton X-100 were necessary in the extraction buffer to recover maximum activity from the stem sections, as was reported for apple fruits [4]. For the time course of induction by ethylene, we found that addition of 1 mM ACC to the extraction buffer was necessary to recover maximum enzyme activity. The addition of ACC to the extraction buffer for the IAA-treated tissue did not increase the recoverable ACC oxidase activity. We believe that the ACC stabilized the enzyme during extraction and that IAA-treated tissue produced sufficient amounts of ACC to stabilize ACC oxidase without the addition of ACC to the extraction buffer.

The activity assays consisted of 200 μl enzyme extract, 50 μl of 40 mM ACC, 50 μl of 2 mM $FeSO_4$, and 1.7 ml of the extraction buffer in a total volume of 2 ml. The reaction mixtures were shaken at 30 °C for 1 h in 9 ml screwcap tubes, each fitted with a teflon-coated septum (Fischer Scientific, Pittsburgh, PA). At the end of this time period, 1 ml of the headspace was removed and analyzed by gas chromatography. As reported previously [4], the addition of CO_2 to the reaction mixture greatly stimulates ACC oxidase activity. However, since the level of CO_2 within the tissue was not known, and since ACC oxidase activity could be assayed without the addition of CO_2, all experiments were performed at ambient CO_2 concentrations. The *in vitro* activity was saturated at 1 mM ACC, required ascorbate and $FeSO_4$ for maximal activity, was inhibited by Co^{2+}, and was completely abolished by 5 min treatment at 90 °C. These results agree with previously established parameters for specific ACC oxidase activity.

ACC synthase enzyme assays

Because of the low ACC synthase activity recovered from pea-stem tissue, it was necessary to modify the extraction procedure from that described by Jones and Kende [8]. Approximately 10 g FW of stem tissue was ground in liquid nitrogen and extracted with 0.5 ml/g FW of buffer (200 mM sodium phosphate pH 8.0, 5 mM DTT, 10 μM pyridoxal 5-phosphate). The slurry was centrifuged at 25000 $\times$ *g* for 20 min at 4 °C. The supernatant was dialyzed overnight against two changes of buffer (10 mM sodium phosphate pH 8.0, 5 μM pyridoxal 5-phosphate) at 4 °C.

The volume of the dialyzed extract was reduced by placing the dialysis bag onto dry polyethylene glycol until 1 ml of extract represented ca. 1 g FW of tissue. In the assay, 2 ml of concentrated extract was combined with 200 μL of 1 M sodium phosphate pH 8.0, and either 50 μl of 0.2 M AdoMet or H_2O for a total volume of 2.25 ml in a 9 ml tube, and the reaction mixture was shaken at 30 °C for 1 h. The ACC produced was determined by the method of Lizada and Yang [12].

Cloning of ACC synthase

To clone the gene for IAA-induced ACC synthase, we used reverse transcription (RT)-polymerase chain reaction (PCR) with total RNA isolated from tissue treated for 2 h with IAA. In the RT reaction, we combined 2 μl of 0.2 μg/μl oligo(dT)$_{15}$ (Pharmacia, Piscataway, NJ), 10.6 μl of 2.5 mM dNTP mixture, 0.4 μl of 40 U/μl RNAsin RNAse inhibitor (Promega, Madison, WI), 1 μl of 200 U/μl M-MLV reverse transcriptase with 4 μl of the supplied 5 × RT buffer (Promega), and 2 μl of 1 μg/μl total RNA for a total volume of 20 μl. This mixture was incubated at 37 °C for 1 h, and the reaction stopped by incubation at 95 °C for 10 min. We used 2 μl of the RT reaction for PCR. Two degenerate oligonucleotide primers, 5′-CTC(GAATTC) ACCAAYCCNTC-NAAYCCNYTRGG-3′ and 5′-CTC(AAGCT-T)ACNARNCCRAARCTYGACAT-3′ based on conserved amino acid sequences (TNPSN-PLG and MSSFGLV, respectively) were synthesized containing *Eco* RI and *Hin*d III restriction sites (shown in parenthesis) at the 5′ ends, respectively, and used for PCR. Thermocycling was performed at 94 °C for 1 min, 42 °C for 1 min, and 72 °C for 1 min, for 35 cycles. The products were digested with *Eco* RI and *Hin*d III, separated by agarose gel electrophoresis, recovered by electrophoresis onto DE-81 paper [18], ligated into pBluescript SK$^-$ (Stratagene, La Jolla, CA), and transformed into INVαF′ cells (Invitrogen, San Diego, CA). Five independent clones were selected for sequencing. The sequences of all five were identical in both strands except for the primer regions. One of these clones (317 bp) was selected for further study. Since we had previously isolated a different ACC synthase cDNA clone (PS-ACS1, unpublished) from the hook region of etiolated pea seedlings, the PCR-generated clone was designated PS-ACS2 (see Fig. 1).

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1   ACTGTTATGGACAGAAACACACTAAGAACTGTCATCACTTTCATCAACGAAAAGCGTATC
    T  V  M  D  R  N  T  L  R  T  V  I  T  F  I  N  E  K  R  I

61  CATCTAATAAGCGACGAAATCTACGCTGCAACGGTTTTTAGCCACCCAAGTTTCATAAGC
    H  L  I  S  D  E  I  Y  A  A  T  V  F  S  H  P  S  F  I  S

121 ATAGCGGAGATAATCGAACATGACACAGACATTGAATGCGACCGTAACCTCGTTCACATA
    I  A  E  I  I  E  H  D  T  D  I  E  C  D  R  N  L  V  H  I

181 GTTTATAGTCTTTCAAAAGTCATGGGATTCCCGGGGTTTAGAGTTGGTATAATATACTCA
    V  Y  S  L  S  K  D  M  G  F  P  G  F  R  V  G  I  I  Y  S

241 TACAACGACACCGTTGTTGATTGCACGCGCAAA
    Y  N  D  T  V  V  D  C  T  R  K
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Fig. 1. Nucleotide and deduced amino acid sequence of ACC synthase cDNA clone PS-ACS2. The sequence does not contain the primers used for PCR (described in Materials and methods). The conserved active site region is underlined.

RNA blot analysis

All RNA isolations were performed as described by Puissant and Houdeline [15]. For ACC oxidase RNA blots, 20 μg of total RNA was separated on a 1.2% agarose-formaldehyde gel. Ethidium bromide staining of the ribosomal bands was used to confirm equal loading of lanes. Gels were transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL). The filters were baked at 80 °C for 1–2 h and prehybridized at 62 °C with 5 × SSPE, 10 × Denhardt's solution, 0.1% SDS, 0.25 mg/ml salmon sperm DNA, and 50% formamide, for at least 4 h. A full-length (1122 bp) pea ACC oxidase clone (pPE8) [14] in pBluescript SK$^-$ (Stratagene) was used to synthesize a ^{32}P-labeled antisense strand RNA probe by using T7 RNA polymerase. Hybridization was performed at 62 °C in the prehybridization buffer. The filters were washed 3 times for 15 min each in 0.2 × SSC, 0.2% SDS, at 62 °C. Autoradiography was performed using Hyperfilm-MP (Amersham) at −80 °C with two amplification screens. The signals were quantified with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

For ACC synthase RNA blots, conditions were as described except that 5 μg of poly(A)$^+$ RNA

was used. The poly(A)$^+$ RNA was isolated using the Mini-Oligo(dT) Cellulose Spin Column kit (5 Prime→3 Prime, Inc., Boulder, CO) according to the manufacturer's instructions. The riboprobe was prepared from PS-ACS2, a 317 bp PCR product (Fig. 1).

Results

Cloning of an IAA-inducible ACC synthase

To obtain a probe for measuring ACC synthase transcript levels, we used RT-PCR to clone an ACC synthase cDNA fragment (PS-ACS2) from the first internode of etiolated pea seedlings treated for 2 h with IAA. This cDNA contained the nucleotide sequence encoding the active site of ACC synthase. PS-ACS2 (Fig. 1) has 79% and 67% nucleotide identity with the corresponding regions of two IAA-induced ACC synthase cDNA clones from etiolated mung bean hypocotyl sections [2, 10].

Ethylene increases ACC oxidase activity and transcript levels

Schierle *et al.* [20] found that ethylene pretreatment of stem sections from etiolated pea seedlings stimulated *in vivo* ACC oxidase activity. We expanded upon these experiments using intact seedlings for treatments and an *in vitro* enzyme assay. ACC oxidase transcript levels and enzyme activity increased with the duration of exposure to 40 μl/l ethylene (Fig. 2). ACC oxidase transcript and enzyme activity were present at low but detectable levels in the untreated tissues. After 12 h of exposure to ethylene, the transcript level had increased almost 100-fold, and the enzyme activity had increased about 10-fold. The changes measured in *in vitro* enzyme assays were consistent with those seen *in vivo* when the conversion of exogenous ACC (1 mM) to ethylene was measured in stem sections (data not shown). The increase in ACC oxidase activity was not a result of handling during treatment or of enclosing seedlings in desiccators because neither the transcript level nor the activity of the enzyme increased in the control treatments (Fig. 2). Since ethylene can cause the accumulation of ACC synthase activity in some tissues [24], it could be argued that ethylene induced ACC oxidase activity by increasing the availability of ACC. To address this possibility, we examined the levels of ACC synthase transcript, ACC synthase enzyme activity, and ACC during ethylene treatment. We detected no changes in any of these levels during the treatment of the seedlings with ethylene (data not

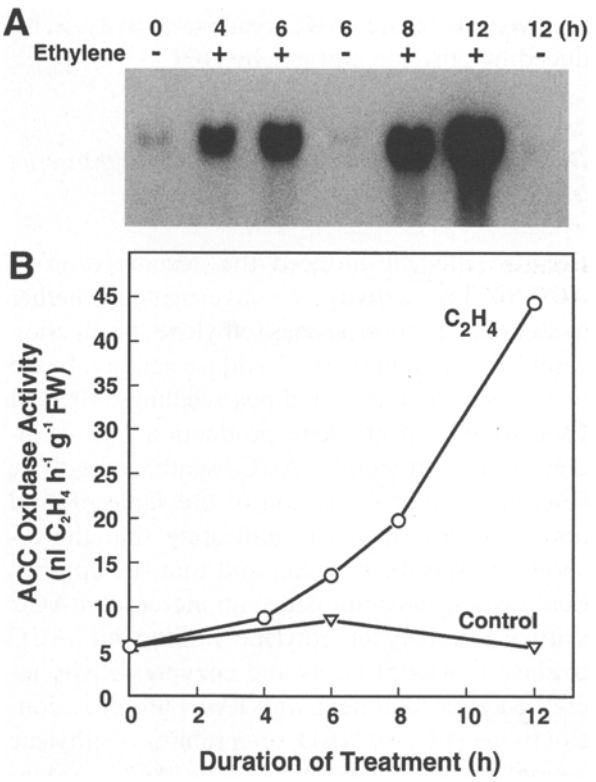

Fig. 2. Effect of ethylene on ACC oxidase transcript abundance (A) and enzyme activity (B). At 0 h, intact seedlings were placed in desiccators with (○) or without (△) 40 μl/l ethylene. At the times indicated, the seedlings were removed from the desiccators, and 2-cm sections from the first internode were isolated and used for RNA isolation and enzyme assays. For RNA blot analysis (A), 20 μg of total RNA was separated on a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane, and probed with a ^{32}P-labeled antisense RNA strand prepared from pPE8 [12]. The experiment was performed four times with similar results.

shown). Therefore, ACC oxidase activity is induced by ethylene and not by ACC.

IAA increases ACC oxidase activity and transcript levels via ethylene

Because ethylene induced the accumulation of ACC oxidase activity, we investigated whether a stimulus that promotes ethylene production would also stimulate ACC oxidase activity. In the first internode of etiolated pea seedlings, 100 μM IAA stimulated ethylene production via an increase in extractable ACC synthase activity (Fig. 3). A control solution of the same pH did not cause this increase, indicating that the response is specific to auxin and that the application method does not cause an increase in ACC synthase activity or ethylene production. ACC oxidase transcript levels and enzyme activity increased after treatment with IAA but not in control tissue (Fig. 4). NBD, an inhibitor of ethylene action, prevented the increase in ACC oxidase transcript and enzyme activity levels after treatment with IAA (Fig. 4). NBD did not prevent the IAA-induced accumulation of ACC synthase activity (data not shown), showing that NBD did not prevent the response to IAA. To determine if NBD prevented the increase in ACC oxidase activity non-specifically, we added saturating levels of ethylene (300 μl/l) to the IAA + NBD treatment. Ethylene restored the ACC oxidase activity and transcript levels in the presence of NBD (Fig. 5), supporting earlier evidence that NBD

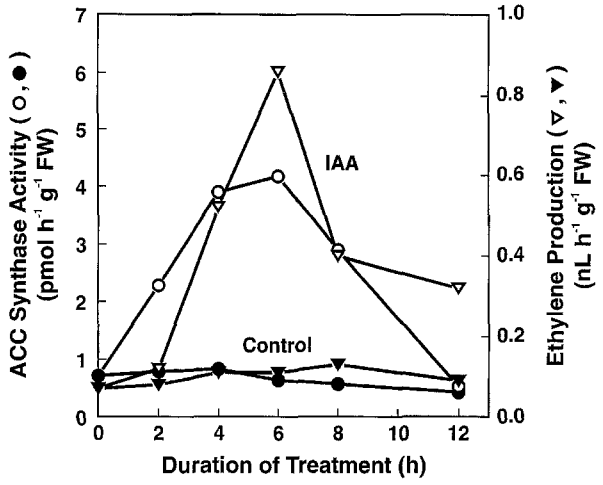

Fig. 3. Effect of IAA on ACC synthase activity and rate of ethylene production in the first internode of etiolated pea seedlings. At 0 h, seedlings were sprayed with solutions containing 100 μM IAA (○, ▽) or no IAA (●, ▼). At the times indicated, 2-cm sections were isolated from the first internode and used for ACC synthase enzyme assays (○, ●) or for measuring ethylene production (▽, ▼). The experiment was performed twice with similar results.

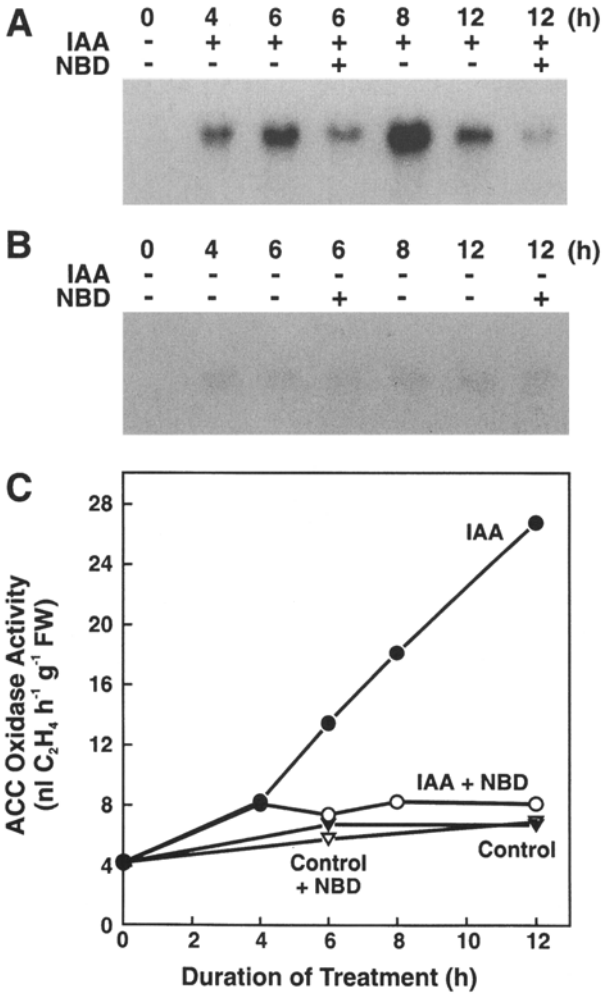

Fig. 4. Effect of IAA and NBD on ACC oxidase transcript abundance (A, B) and enzyme activity (C). Treatments were as described in Fig. 3. Plants were treated with auxin (○, ●) or control solutions (▽, ▼) in the presence (○, ▽) or absence (●, ▼) of NBD. RNA blot analysis was performed as described in Fig. 2. The experiment was performed four times with similar results.

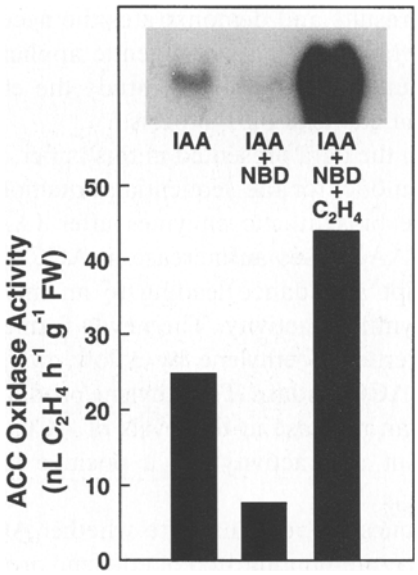

Fig. 5. Competitive effects of NBD and ethylene on ACC oxidase transcript abundance and enzyme activity during IAA treatment. Seedlings were treated with auxin as described in Fig. 3 for 12 h with or without NBD or ethylene. RNA blot analysis was as described in Fig. 2. The experiment was performed twice with similar results.

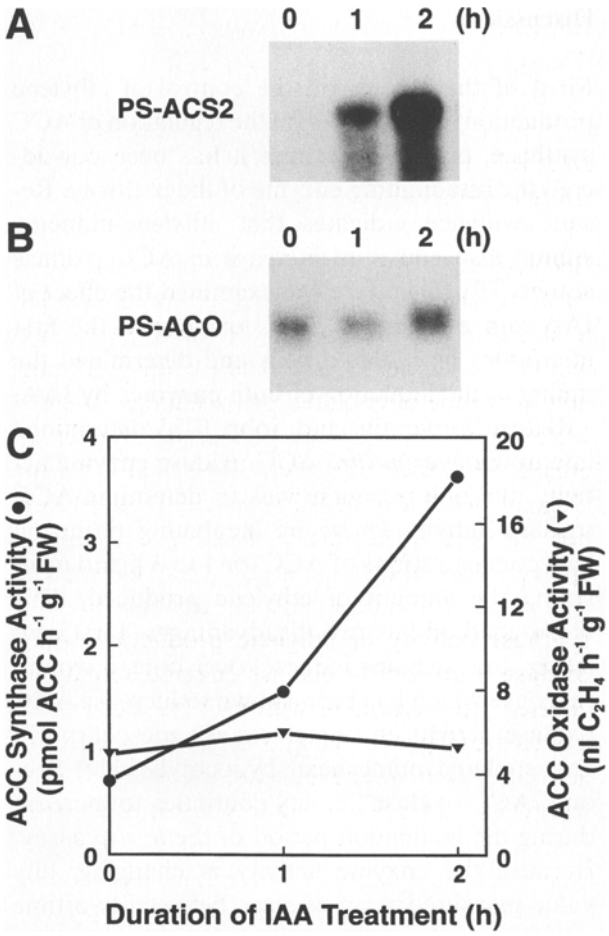

Fig. 6. Comparison of rates of accumulation of ACC synthase (●) and ACC oxidase (▼) transcript and enzyme activity after auxin treatment. Treatment conditions were as described in Fig. 3, and RNA blot analysis as in Fig. 2, except that 5 μg poly(A)$^+$ RNA was used with an ACC synthase antisense RNA strand made from pPS-ACS2 as a probe. The experiment was performed three times with similar results.

acts specifically as a competitive inhibitor of ethylene action. The difference in transcript abundance between the IAA and the IAA + NBD + ethylene treatments (Fig. 5) was not surprising. In the IAA treatment, the amount of transcript reached a maximum at 8 h and decreased by 12 h (Fig. 4A), presumably because IAA-induced ethylene production had ceased (note the pattern of ethylene production in Fig. 3). In the presence of ethylene, the ACC oxidase transcript level continued to increase during the entire period of treatment (Fig. 2). These results show that IAA increases the levels of ACC oxidase transcript and activity via ethylene.

The evidence that IAA treatment resulted in an increase in ACC synthase activity and that the ethylene produced caused an increase in ACC oxidase activity indicated that the ethylene biosynthetic enzymes are sequentially induced. If so, the increase in ACC synthase activity should be detectable before the increase in ACC oxidase activity. Figure 6 shows the changes in ACC synthase and ACC oxidase transcript levels and activities during the first 2 h of IAA treatment. The levels of ACC synthase transcript and enzyme activity increased within the first hour and continued to increase over the second hour (Fig. 6A and C). ACC oxidase transcript, however, did not begin to increase until 2 h after the start of IAA treatment (Fig. 6B). ACC oxidase activity did not increase during the first 2 h (Fig. 6C), although it did increase about 2-fold by 4 h (Fig. 4C).

Discussion

Most of the studies on the control of ethylene production have focused on the regulation of ACC synthase, primarily because it has been considered the rate-limiting enzyme of the pathway. Recent evidence indicates that ethylene-inducing stimuli also cause an increase in ACC oxidase activity [9]. Therefore, we examined the effect of IAA and ethylene on ACC oxidase in the first internodes of etiolated peas and determined the timing of the induction of both enzymes by IAA.

Before Ververidis and John [23] determined how to recover *in vitro* ACC oxidase enzyme activity, the only recourse was to determine ACC oxidase activity *in vivo* by incubating tissue on high concentrations of ACC for 1 to 3 h and measuring the amount of ethylene produced. This *in vivo* method has two disadvantages. First, isolating stem sections induces, over time, a wound response which has been shown to increase ACC oxidase activity in winter squash mesocarp [7] and etiolated mung bean hypocotyls [11]. Second, ACC oxidase activity continues to increase during the incubation period of the *in vivo* assay. Because the enzyme activity is changing, any value measured is an average between two time points and cannot be attributed to any one time point on a curve. This complicates comparisons between activity levels and transcript abundance because the RNA is isolated at specific time points.

To exclude the effects of wounding, it was important to treat intact seedlings with the auxin solutions. Previously, IAA induction studies involved incubating tissue sections on IAA solutions that often contained a cytokinin to maximize changes in the normally low ACC synthase activity [10, 25]. It is now apparent, however, that this type of treatment can cause both wound-induced and IAA-induced transcription of different ACC synthase genes, with unknown effects resulting from the benzyladenine treatment. The multiple gene products could contribute to changes in ACC synthase activity and ethylene production. The obvious complexity of these types of experiments complicates the interpretation of results and demonstrates the necessity of using intact seedlings with gentle application of test solutions to specifically study the effects of auxin on ethylene biosynthesis.

From the data presented in this paper, we propose a model for the sequential induction of the ethylene biosynthetic enzymes after IAA treatment. IAA causes an increase in ACC synthase transcript abundance leading to an increase in ACC synthase activity. The newly formed ACC is converted to ethylene by a low, constitutive level of ACC oxidase. The ethylene produced then causes an increase in the levels of ACC oxidase transcript and activity via a positive feedback loop.

The question remains as to whether ACC oxidase is a rate-limiting step of ethylene production and whether the increased ACC oxidase activity contributes to the higher level of ethylene production. The rate of ethylene production increases steeply between 2 and 4 h after IAA treatment (Fig. 3), which is approximately the same time when ACC oxidase activity begins to increase. While this result indicates that the increase in ACC oxidase activity and the increase in ethylene production rate are related, the evidence is correlative. We are continuing experiments to examine the role of ACC oxidase in regulating the rate of ethylene production.

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