Asymmetric Responsiveness to Ethylene Mediates Cell Elongation in the Apical Hook of Peas

Scott C. Peck, a,1 Katharina Pawlowski, b,2 and Hans Kende a,3
a Michigan State University–Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824-1312
b Department of Molecular Biology, Agricultural University, 6703 HA Wageningen, The Netherlands

The apical hook of dark-grown dicotyledonous seedlings is a protective structure resulting from an inhibition of cell elongation on the inner portion of the hook. This differential growth response is mediated by ethylene. Expression of the gene encoding 1-aminocyclopropane-1-carboxylate oxidase (ACO), the terminal enzyme in ethylene biosynthesis, is induced by ethylene via a positive feedback loop. Therefore, the ACO transcript can serve as a molecular marker for both ethylene formation and ethylene responsiveness. We examined the distribution of ACO mRNA of pea, Ps-ACO1, and of ACO enzyme activity in the apical hook of etiolated pea seedlings. In situ hybridization showed that cells on the inner, concave side of pea hooks accumulated more Ps-ACO1 mRNA than did cells on the outer, convex side. The distribution of ACO enzyme activity followed the same pattern. A direct correlation was observed between the cellular distribution of Ps-ACO1 mRNA, ACO enzyme activity, and the inhibition of cell elongation. Pea seedlings treated with a saturating concentration of ethylene still accumulated higher levels of the Ps-ACO1 transcript on the inner side of the apical hook, demonstrating an increased responsiveness to ethylene in this tissue. These results indicate that an asymmetrically distributed component of the ethylene signal transduction pathway mediates hook formation. Based on existing genetic evidence, we propose that this component is downstream from the serine/threonine protein kinase CTR1.

INTRODUCTION

During germination of a dicotyledonous plant, such as pea, the shoot emerges from the seed with a hook-shaped structure that protects the apical meristem and first leaves as the seedling pushes through the soil. The apical hook is maintained until the seedling reaches the light, at which time the hook opens, the leaves expand, and the photosynthetic apparatus differentiates. Formation of the apical hook results from the continuous movement of newly formed cells through a complex pattern of cell elongation. As the cells exit the apical meristem, those on the inner side of the hook elongate more slowly than do those on the outer side, resulting in curvature of the stem (Rubinstein, 1971). To form a straight stem below the hook, this inhibition is released, and the cells on the inner side expand rapidly to match the length of the outer cells. Ethylene is known to inhibit growth of vegetative tissues, and both physiological (Palmer, 1985) and genetic (Ecker, 1995; Bleecker and Schaller, 1996) evidence shows that ethylene mediates the inhibition of cell elongation on the inner side of the hook.

Ethylene could regulate differential growth in the hook region via two mechanisms that are not mutually exclusive. A higher local concentration of ethylene may inhibit elongation on the inside of the hook, or cells on the inner side may be more responsive to ethylene than those on the outer side. Although some indirect evidence exists for slightly higher levels of ethylene production on the inner side of the hook (Schwark and Bopp, 1993), the difficulty of accurately measuring local ethylene concentrations has greatly impeded our understanding of hook formation. Even though genes encoding components of the ethylene biosynthetic and signal transduction pathways have been cloned, localization of the corresponding transcripts in the apical hook has not been reported.

We have used the transcript (Ps-ACO1) encoding the terminal enzyme in ethylene biosynthesis, 1-aminocyclopropane-1-carboxylate oxidase (ACO), as a molecular marker to determine the cellular pattern of ethylene production and/or responsiveness in the apical hook of etiolated pea seedlings. In pea and mung bean seedlings, the abundance of ACO mRNA is regulated by the endogenous level of ethylene via a positive feedback loop (Peck and Kende, 1995; Kim et al., 1997). ACO enzyme activity and transcript levels increase in
pea tissues treated with ethylene (Schierle et al., 1989; Peck and Kende, 1995). Similar increases have also been measured in tissues that are wounded (Holdsworth et al., 1987; Hyodo et al., 1993; Kim and Yang, 1994) or treated with indole-3-acetic acid (IAA; Peck and Kende, 1995). Both of these stimuli are known to induce ethylene production, IAA- and wound-induced increases in ACO enzyme activity and transcript levels are blocked by 2,5-norbornadiene, an inhibitor of ethylene action. This shows that ACO expression increases in response to endogenously produced ethylene and not in response to the initial stimulus, that is, wounding or auxin (Hyodo et al., 1993; Peck and Kende, 1995). It is therefore possible to determine which cells in the apical hook produce ethylene and/or respond to it by examining the distribution of ACO mRNA using in situ hybridization.

In this work, we show that Ps-ACO1 mRNA is more abundant in the inner than the outer portion of the apical hook and that the distribution of Ps-ACO1 mRNA correlates with the inhibition of growth that results in hook formation. By examining ethylene-treated seedlings, we also show that the inner portion of the apical hook is more responsive to ethylene than is the outer portion. The results presented in this study, in conjunction with existing genetic data, indicate that formation of the apical hook is regulated by asymmetric distribution or activity of a component of the ethylene signal transduction pathway acting downstream of the serine/threonine protein kinase CTR1.

RESULTS

ACO Is a Molecular Marker for Ethylene Responsiveness

With a 2-hr lag time, the increase in the level of the Ps-ACO1 transcript followed the pattern of IAA-induced ethylene production in both apical hooks and stems of etiolated pea seedlings (Figures 1A and 1B). The relative increase of Ps-ACO1 mRNA was lower in the apical hook than in the stem, because the basal level of ethylene production in the hook region is higher than in other parts of the etiolated seedling (Schierle et al., 1989). This observation correlates with higher levels of Ps-ACO1 mRNA in the untreated hook tissue. The lag time of Ps-ACO1 mRNA accumulation confirms that the expression of the transcript is responding to changes in ethylene production and not vice versa. Thus, the level of Ps-ACO1 mRNA in regions of the apical hook can be used as a molecular marker for ethylene synthesis and/or responsiveness to ethylene.

Ps-ACO1 Is Asymmetrically Localized in the Apical Hook

The pattern of Ps-ACO1 mRNA accumulation is asymmetric in the apical hook of air-grown seedlings (Figure 2A). The transcript is more abundant on the inner side of the hook and extends farther toward the stem than on the outer side. This distribution of the Ps-ACO1 transcript agrees well with the role of ethylene as an inhibitor of cell elongation (reviewed in Yang and Hoffman, 1984). Cells emerging from the apex into the hook (Figure 3A, zone 1) have the highest level of Ps-ACO1 mRNA (Figure 2A) and are smallest in size (Figure 3B). On both the outer and inner sides of the hook, a decrease in Ps-ACO1 mRNA coincides with an increase in cell expansion (Figure 3B, zones 3 to 6 on the outer side and zones 6 to 9 on the inner). The decrease in Ps-ACO1 labeling did not result from the dilution of an equal amount of labeling as the cells expanded. Using magnified images of Figure 2A, we
Figure 2. Localization of Ps-ACO1 mRNA in the Apical Hook of Etiolated Pea Seedlings.

Apical hooks were isolated from etiolated 5- to 6-day-old seedlings. In (A) to (C), the apex (a) is toward the right side and the straight stem (s) is on the left. At left are dark-field images, and at right are bright-field images.

(A) Hook isolated from an air-grown seedling. Hybridization was performed with a 35S-labeled antisense strand of Ps-ACO1. Fifteen hooks from two independent fixation and hybridization experiments were examined, and a similar pattern of Ps-ACO1 distribution was found in each.

(B) Hook isolated from a seedling treated with 40 µL L⁻¹ of ethylene. Hybridization was as given in (A). Ten ethylene-treated hooks from two independent fixation and hybridization experiments were examined, and a similar pattern of Ps-ACO1 distribution was found in each.

(C) Hook isolated from an air-grown seedling. Hybridization was performed with a 35S-labeled sense strand of Ps-ACO1. The pink spots are due to autofluorescence of the cell wall.

Bar at right in (A) = 0.45 mm for images in (A) to (C).
determined that the number of silver grains per cell is greater on the inner side of the hook than on the outer side (Figure 3C). The in situ hybridization results therefore reflect true differences in cellular Ps-ACO1 mRNA content. Thus, there is a direct correlation between asymmetric ethylene production and/or responsiveness, which is represented by Ps-ACO1 transcript abundance, and asymmetric inhibition of cell elongation resulting in the formation of the apical hook.

To confirm the in situ localization results by an independent method, we isolated four regions from the inner and outer sides of apical hooks for in vitro ACO enzyme assays. As shown in Figure 4, the distribution of enzyme activities in these four regions is in agreement with the pattern of mRNA distribution observed in Figure 2A.

### The Inner Side of the Hook Is More Responsive to Ethylene Than Is the Outer Side

The asymmetry in Ps-ACO1 mRNA accumulation (Figure 2A) can be explained by higher production of ethylene in the inner portion of the hook, by a greater responsiveness to uniform ethylene production across the hook, or by a combination of the two. To investigate the possibility of asymmetry in ethylene responsiveness, we examined seedlings treated with ethylene for 18 hr (Figure 2B). A saturating ethylene concentration in the atmosphere surrounding the seedling would abolish any gradient established by asymmetric ethylene production. As expected, Ps-ACO1 mRNA levels were higher in the ethylene-treated seedlings than in the air-grown ones. Even in the presence of saturating concentrations of ethylene, the outer portion of the hook accumulated less Ps-ACO1 mRNA than did the inner portion, especially in the region extending toward the straight stem (Figure 2B). We quantified the number of silver grains per cell in three regions of the ethylene-treated hook, namely, at the apical end (Figure 3C, zone 2) that contains recently formed cells, at the crest of the hook (zone 5), and at the basal end (zone 7). We found that differential responsiveness to ethylene develops as the cells progress through the hook. In the crest region, the cells on the inner side of the ethylene-treated hook accumulated 50% more Ps-ACO1 mRNA than did cells on the outer side. These results confirm that the inside of the hook is more responsive to ethylene than is the outside.

In some dicotyledonous seedlings, for example, Arabidopsis, prolonged treatment with ethylene causes the apical hook to close more tightly. As seen in Figure 2B, this is not the case in pea seedlings. Cell sizes were reduced on both sides of the hook in ethylene-treated seedlings, but cells on the outer side were still longer than those on the inner side (Figure 3B). By marking the inner and outer sides of the hook with India ink, we found that growth on both sides of the ethylene-treated hooks was inhibited by >90% compared with apical hooks grown in air (results not shown). By inhibiting the movement of cells through the hook, ethylene treat-
ment maintained hook closure without inducing exaggerated hook formation.

Ps-ACO1 mRNA Does Not Accumulate in the Epidermal Layers

Ps-ACO1 mRNA accumulation was found in all cells of the cortex but was quite low or undetectable in the central portion of the stem (Figure 2). Under higher magnification, it was also found that the transcript was not present in the epidermal cells, even after prolonged ethylene treatment (Figures 5A and 5B). The absence of Ps-ACO1 mRNA in the epidermis on both sides of the apical hook is consistent with an earlier report that epidermal cells do not contain the enzymes of the ethylene biosynthetic pathway (Todaka and Imaseki, 1985).

DISCUSSION

Because ethylene increases the level of Ps-ACO1 mRNA and ACO enzyme activity in pea stems via a positive feedback loop (Peck and Kende, 1995), the Ps-ACO1 transcript is a molecular marker for both ethylene synthesis and responsiveness to ethylene. We localized expression of Ps-ACO1 mRNA to determine which cells in the apical hook of etiolated pea seedlings are producing ethylene and/or responding to it. Our results show that the Ps-ACO1 transcript is asymmetrically distributed in the hook, with a higher level of expression in the inner than in the outer portion. A strong correlation exists between the localization of Ps-ACO1 mRNA and the inhibition of cell elongation, resulting in formation of the hook. This is consistent with previous evidence that ethylene is an inhibitor of growth in vegetative tissues (reviewed in Yang and Hoffman, 1984).

In air-grown seedlings, the accumulation of the Ps-ACO1 transcript may indicate both increased ethylene synthesis and increased responsiveness to ethylene. However, in seedlings exposed to saturating concentrations of ethylene, only increased responsiveness to ethylene can explain the accumulation of Ps-ACO1 mRNA on the inner side of the hook. Ultimately, asymmetry in ethylene responsiveness rather than asymmetry in ethylene production may mediate the formation of the apical hook.

Genetic evidence supports the conclusion given above and also provides insight into which component of the ethylene

Figure 4. Distribution of ACO Enzyme Activity.

Hooks were sectioned into four parts, and in vitro ACO enzyme assays were performed using extracts from these sections (units are in nanoliters of ethylene per hour per gram fresh weight of tissue). The apex is on the right and the stem on the left side of each hook. This experiment was performed three times with a similar pattern seen for each.

Figure 5. Ps-ACO1 mRNA Does Not Accumulate in the Epidermal Cells.

High-magnification images were taken of the hook shown in Figure 2B. The arrows in (A) and (B) point to the epidermal cell layer. The bar in (B) = 40 μm for (A) and (B).

(A) Dark-field microscopy.
(B) Bright-field microscopy.
response system may be localized asymmetrically. Elements of the ethylene signal transduction pathway, including the receptor(s) for ethylene, have been identified in Arabidopsis (reviewed in Ecker, 1995; Bleecker and Schaller, 1996). All evidence to date indicates that ethylene responses converge in a single pathway that includes a serine/threonine kinase encoded by a single-copy gene, CTR1 (Kieber et al., 1993). When the CTR1 kinase is active, it blocks ethylene responses. A null mutation in CTR1 results in constitutive activation of ethylene responses, just as would occur in ethylene-treated seedlings, and the mutant has a tightly closed apical hook. Because this null mutation uncouples the ethylene receptor(s) from ethylene responses and because the apical hook still forms, it can be argued that the asymmetry in the ethylene signaling pathway is downstream from CTR1 (Figure 6). Accordingly, asymmetry of all factors upstream of CTR1—including ethylene perception at the level of the receptors and increased local ethylene production—is not likely to be the primary reason for hook formation.

The question still remains as to how ethylene mediates the asymmetry in growth that results in hook formation. It may do so by altering the distribution of the growth-promoting hormone auxin. An Arabidopsis mutant, hls1, does not form an apical hook, even in a double mutant made with ctr1 (Roman et al., 1995). The phenotype of the mutant mimics that seen when wild-type seedlings are treated with auxin transport inhibitors (Lehman et al., 1996). From these results, it appears that redistribution of auxin may be involved in hook formation and that the protein encoded by the ethylene-regulated gene HLS1 may mediate this response.

Our results, together with existing genetic data, indicate that an asymmetry in the ethylene signal transduction pathway mediates the differential inhibition of growth, resulting in formation and maintenance of the apical hook. They also provide a focus for the identification of the asymmetric component of the ethylene signal transduction pathway that is likely to be downstream of CTR1 but upstream of the factors regulating the transcription of Ps-ACO1.

METHODS

Plant Material and Hormone Treatments

Pea seeds (Pisum sativum cv Alaska; Cliston Seed Co., Faison, NC) were imbibed overnight in aerated tap water. Seedlings were grown in vermiculite at 25°C in darkness for 6 to 7 days, and all manipulations were performed under a green safelight (530 to 590 nm). For ethylene treatments, seedlings were enclosed in 9-liter desiccators fitted with injector ports that were sealed with rubber serum vial caps. Ethylene was injected into the desiccators, and the desired concentration was verified by gas chromatography. Solutions containing 100 μM indole-3-acetic acid (IAA), 0.05% ethanol, and 0.05% Tween 20, with the pH adjusted to 6.0 with dilute NaOH, were gently sprayed as a fine mist onto the seedlings, using a spray bottle. Control plants were sprayed with the same solution but without IAA.

Measuring the Rate of IAA-Induced Ethylene Production

At the indicated times after the start of the IAA treatment, the entire apical hook minus the plumule, or 2-cm sections from the first (lowest) internode, were isolated and enclosed in 4-mL tubes fitted with serum vial caps. After 30 min, 1 mL of headspace was withdrawn with a syringe for determination of ethylene concentration by gas chromatography. These 30 min are within the lag phase of wound-induced ethylene synthesis in etiolated pea stems (Saltveit and Dilley, 1978).

RNA Gel Blot Analysis

All RNA isolations were performed as described by Puissant and Houdebine (1990), and RNA gel blot analysis was as previously described (Peck and Kende, 1995). The hybridization signals were quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) analysis.

In Situ Localization of Ps-ACO1 mRNA

Fixation of the tissue and in situ hybridization were performed as previously described (Van de Wiel et al., 1990). A full-length 1-aminocyclopropane-1-carboxylate oxidase (ACO) clone from pea, pPE8 (Peck et al., 1993), was used to synthesize a 35S-labeled antisense strand Ps-ACO1 RNA probe using T7 RNA polymerase or a sense strand probe using T3 RNA polymerase. The radioactive signal was detected using Amershams emulsion LM-1. Overlapping photographs were taken under dark or bright field and merged into a single image by using Adobe Photoshop (Adobe, Mountain View, CA).

Cross-hybridization of the probe for Ps-ACO1 with transcripts of other ACO genes was deemed unlikely. Screening of the cDNA library yielded only clones corresponding to Ps-ACO1. Further attempts to clone another ACO cDNA from apical hooks and other parts of the stem by reverse transcription–polymerase chain reaction were unsuccessful. These results are consistent with the findings that only a single ACO gene is expressed in vegetative tissues of tomato (Barry et al., 1996) and rice (Mekhedov and Kende, 1996) and indicate that Ps-ACO1 is the predominant if not the only ACO gene expressed in the apical hook.
In Vitro ACO Enzyme Assays

Enzymes assays were performed as described by Peck and Kende (1995) with the addition of 30 mM NaHCO₃ to the reaction mixture.

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