

Regulation of differential growth in the apical hook of *Arabidopsis*

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Accepted 28 May; published on WWW 19 July 1999

SUMMARY

Arabidopsis seedlings develop a hook-like structure at the apical part of the hypocotyl when grown in darkness. Differential cell growth processes result in the curved hypocotyl hook. Time-dependent analyses of the hypocotyl showed that the apical hook is formed during an early phase of seedling growth and is maintained in a sequential phase by a distinct process. Based on developmental genetic analyses of hook-affected mutants, we show that the hookless mutants (*hls1*, *cop2*) are involved in an early aspect of hook development. From time-dependent analyses of ethylene-insensitive mutants, later steps in hook maintenance were found to be ethylene sensitive. Regulation of differential growth was further studied through examination of the spatial pattern of expression of

two hormone-regulated genes: an ethylene biosynthetic enzyme and the ethylene receptor *ETR1*. Accumulation of mRNA for *AtACO2*, a novel ACC (1-aminocyclopropane-1-carboxylic acid) oxidase gene, occurred within cells predominantly located on the outer-side of the hook and was tightly correlated with ethylene-induced exaggeration in the curvature of the hook. *ETR1* expression in the apical hook, however, was reduced by ethylene treatment. Based on the expression pattern of *ETR1* and *AtACO2* in the hook-affected mutants, a model for hook development and maintenance is proposed.

Key words: Differential growth, Ethylene, *HLS*, *ETR1*, *AtACO2*, *Arabidopsis thaliana*

INTRODUCTION

Mechanisms for the protection of cells that are essential for survival have evolved in multicellular organisms. In animals, layers of protective tissues surround indispensable cells, such as stem cells. Plants also possess such mechanisms to protect meristematic cells. In dicot plants, a unique mechanism has evolved to protect the delicate shoot meristem while the seedling makes its way through the soil towards the soil surface (Darwin and Darwin 1881). During germination of the seedling, a hook-like structure is formed at the apical part of the hypocotyl or epicotyl. The apical hook is maintained until the seedling emerges and upon exposure to light, the hook opens. The process is irreversible; once the apical hook has opened it can not reform.

The establishment of the apical hook is orchestrated by the differential elongation rates of cells within the structure. It can be considered as a 'standing wave' of growth; cells 'flow' along the hypocotyl from the apex of the hook towards the basal part of the hypocotyl hook while the curvature remains fixed (Fig. 1). Silk and Erickson (1978) provided a mathematical description of the growth rate of cells in the hypocotyl hook in etiolated lettuce seedlings. Detailed kinematic analysis indicated that the elongation rate of cells on the outer edge of the hook exceeds that of those on the inner edge when cells are apical to the hook midline. Once they have passed through the apex of the hook, the growth rate of cells on the inside edge exceeds that of cells on the outer edge of

the hook, and the hypocotyl straightens. During opening of the apical hook, cells at the inner edge elongate throughout the straightening process (Silk and Erickson, 1978).

The coordination of differential cell growth in the apical hook is regulated by several plant hormones, of which auxin and ethylene have been extensively studied (Ecker and Theologis, 1994; Lehman et al., 1996). Seedlings germinated in the presence of auxin exhibit no curvature of the apical hypocotyl (Schwark and Schierle, 1992). Indeed, the *sur1/alf1/hls3* mutant, which accumulates high levels of auxin, shows a hookless phenotype (Boerjan et al., 1995; Celenza et al., 1995; Lehman et al., 1996). On the other hand, seedlings germinated in the presence of auxin transport inhibitors, such as N-1-naphthylphthalamic acid (NPA) or 2,3,5-triiodobenzoic acid (TIBA), also show no curvature of the apical hypocotyl (Schwark and Schierle, 1992; Lehman et al., 1996). Etiolated seedlings of *rcn1*, a NPA resistant mutant, show reduced curvature of the apical hook (Garbers et al., 1996). These studies suggest that an appropriate positional concentration of auxin is required for the precise coordination of differential growth within the apical hook.

A role for ethylene in differential growth in the *Arabidopsis* hypocotyl has also been established (reviewed by Ecker, 1995). *Arabidopsis* seedlings germinated in the presence of ethylene show exaggerated curvature of the apical hook. An exaggeration in the curvature of the apical hook is also observed in *ethylene overproduction (eto)* and *constitutive ethylene-response (ctr1)* mutants (Guzman and Ecker, 1990).

Ethylene-insensitive (ein) seedlings exhibit a long root and an elongated hypocotyl with reduced apical hook when grown in ethylene. Seven *ein* loci had been identified and their epistatic relationships with respect to *ctr1* have been established (Roman et al., 1995). The genetic relationships between the ethylene transduction mutants are further supported by molecular analyses of the cloned genes (Chao et al., 1997; Hua and Meyerowitz, 1998, and references therein).

In addition to the *ein* mutants, mutants in which ethylene insensitivity is restricted to the apical hook have also been characterized (Guzman and Ecker, 1990; Lehman et al., 1996). In these mutants, the apical hypocotyl is hookless (*hls*) even in the presence of ethylene. Based on the analysis of auxin-response genes, it has been suggested that the *HLS1* gene product modulates the localization or sensitivity to auxin in the apical hypocotyl (Lehman et al., 1996). The *cop2/hls2/amp1* mutant (Hou et al., 1993; Lehman et al., 1996) contains increased cytokinin levels (Chaudhury et al., 1993), further suggesting that the appropriate concentration or ratio of several hormones is required for the proper development of the apical hook.

The apical hook is completely dispensable for plant growth and survival in the laboratory. Thus, this structure provides a superb model system for developmental genetic analysis of the role of hormones in regulation of differential cell elongation. We analyzed the processes of hook establishment and maintenance in wild-type *Arabidopsis* and hook-affected mutant seedlings. The results indicate that the process of hook development can be resolved into at least two sequential phases. The examination of several ethylene-responsive genes, *AtACO2* and *ETR1* whose expression was spatially restricted in the apical hook, further supports this model.

MATERIALS AND METHODS

Arabidopsis strains and growth conditions

All mutants used were in the Colombia (Col) ecotype. AT plates were made as described (Guzman and Ecker, 1990), except vitamins were omitted from the medium. Seeds were plated on AT medium using AT-top agar. After cold treatment at 4°C for 3 days, the plates were incubated in the dark at 24°C for growth experiments. For ethylene treatment, a continuous flow of 10 ppm/l of air was delivered to enclosed seedlings. When indicated 10 μM ACC was included in the AT medium.

Genetic analysis

Double mutants were constructed by crossing *hls1-1* and *etr1-1* or *ein2-5*, *cop2* and *etr1-1* or *ein2-5*. Seeds were harvested from individual F₁ plants. The phenotypes of F₂ etiolated seedlings were scored 2 days after germination in the presence of ethylene or ACC. The double mutants exhibited an elongated root phenotype as seen in the *ein* mutant, and a hookless apical hypocotyl characteristic of the *hls* mutant.

Time-lapse photography

Seedlings germinated for 24 hours in darkness at 24°C were marked with a permanent VWR Lab Marker, with an extra fine tip. Photographs were taken under a dissecting microscope (Olympus SZH) using a green safe filter with TMX Kodak film at 2-hour intervals over an 8-hour period. During this time seedlings were kept at 22°C. Since seedling germination is not fully synchronized after 3 days of vernalization, the time of seedling growth, as shown in Figs

1 and 2, was estimated, based on a direct comparison of seedling root length ($n=30$).

Whole-mount in situ hybridization

Three-day-old seedlings were used for whole-mount in situ hybridization experiments. The protocol was modified from the original of de Almeida Engler et al. (1994). Seedlings were placed in fixation buffer, containing PBT (PBS; 0.1% v/v Tween), 0.08 M EGTA, 5% formaldehyde; 10% DMSO, for 30 minutes under vacuum. Seedlings were washed twice to remove fixation buffer using 100% methanol and four times using 100% ethanol. They were then post-fixed with xylene:ethanol 1:1 for 30 minutes, following by washing twice with 100% ethanol, 100% methanol and methanol:PBT 1:1. Prior to pre-hybridization, seedlings were fixed with 5% formaldehyde in PBT for 30 minutes, followed by washing twice with PBT. To enhance probe and antibody penetration, seedlings were treated with 2% driselase (Koywa Hakko Kogyo CO., LTD. Tokyo Japan) in PBT under vacuum for 12 minutes. Driselase was removed by washing seedlings three times with PBT and the procedure was continued according to de Almeida Engler et al. (1994). Prior to hybridization, seedlings were incubated with hybridization buffer at 52-55°C for 1 hour and a partial hydrolyzed DIG-labeled riboprobe (2 μg/ml) was added to the hybridization buffer containing the seedlings. Hybridization was carried out for 16 hours. The cDNA probes were UTP-DIG labeled according to the kit protocol (Boehringer Mannheim). Riboprobes were partially hydrolyzed to a size range of 200-100 bp length as described by Cox and Goldberg (1989). DIG-labeled oligo-dT hybridization was carried out at 37°C. The seedlings were washed to remove non-hybridized probe and pre-absorption of the anti-DIG antibody (Boehringer Mannheim) was carried out as described by de Almeida Engler et al. (1994). Anti-DIG antibody was pre-absorbed against 100 μg acetone-fixed plant tissue. The chromogenic reaction was as follows: 1 hour at 37°C followed by 2 hours at room temperature and overnight at 4°C. It was stopped by repeated washing of the seedlings with water. As well as with very heavy staining in the hook and root, staining was sometimes seen in vascular cells. As staining in the vascular cells was absent in many seedlings, we believe that it is due to over staining.

Cloning of *AtACO2*

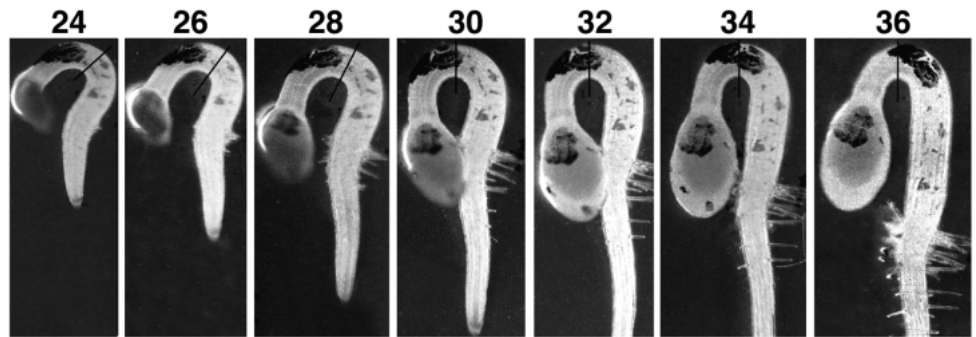
The EI305 cDNA was isolated by differential screening of a cDNA library for ethylene-regulated mRNAs (M. Rothenberg and J. R. E., unpublished results). Database searches using this sequence (GenBank accession AE016100) reveal that this cDNA encodes a novel member of the ACC oxidase gene family. Alignment of the predicted amino acid sequence of the *AtACO2* cDNA with known sequences in the database revealed significant similarity with *Brassica oleracea*, X81628; *Brassica juncea*, Q09052; *Petunia hybridia*, Q08507; *Betula pendula*, Y10749; geranium, U07953; *Lycopersicon esculentum*, P05116; muskmelon, *Cucumis melo*, P54847. *AtACO2* was mapped to chromosome one, 107.9 units on the physical map (<http://genome.bio.upenn.edu/>), using high-stringency hybridization conditions (65°C). Southern blot analyses of genomic Colombia DNA with ACO2 cDNA showed at least 5 members of the gene family under low-stringency hybridization conditions (37°C). However under high-stringency conditions, (50% v/v formamide, 52°C), as used for the whole-mount in situ hybridization, the ACO2 probe did not cross hybridize with other ACO genes.

RESULTS

Early events in apical hook development in *Arabidopsis* seedlings

During germination, the seedling pushes itself out of the seed coat via elongation of cells in the root and hypocotyl. During

Fig. 1. Apical hook development during early stages of seedling germination. Wild-type seeds were germinated in air and photographs were taken after 24, 26, 28, 30, 32, 34 and 36 hours. Epidermal cells were marked at 24 hours with black permanent ink. The straight line indicates the hook midline.



germination, the hypocotyl curves while protruding out of the seed coat (Fig. 1: 24). In 1-day-old etiolated seedlings the measured hypocotyl curvature was variable (Fig. 4B). The curved structure covered a large portion of the hypocotyl and was not localized to its apical region (not shown). However, between 24 and 28 hours after imbibition, the hook was 'shifted' to the apical region of the hypocotyl. In addition, the hook midline was positioned parallel to the apical-basal axis of the seedling, and the apical side of the hook was parallel to its basal side (Fig. 1: 28-30). This process can be referred to as the 'formation step' of the apical hook, and was observed in all seedlings. The exact duration of the formation step differed among individual seedlings, possibly reflecting germination variation. After the apical hook formed, the hypocotyl continued to elongate while both the curvature of the hook and the position of the hook midline parallel to the apical-basal axis of the seedling were maintained (Figs 1: 32-36, 2A). At 22°C, this maintenance step extended for about 44 hours. In 4-day-old seedlings, the curvature of the apical hook was reduced and the hook structure started to open (Fig. 4). During opening, the hook midline changed its position relative to the apical-basal axis of the seedling (Figs 2B: 36 and 38, 3B: 70).

Using time-lapse photography cell flow along the hook midline could be studied in seedlings that had been marked on epidermal cells. Apical cells, near the cotyledons, flowed in an apical-basal direction over the hook midline and joined the elongating hypocotyl (Fig. 1 and 2). Flow of cells was observed on both sides of the hook through the hook midline, and proceeded during the formation (Fig. 1: 26-32) and maintenance (Figs 1: 32-36, 2A) steps. Careful analyses of the marked cells showed that during hook maintenance, cells also rotate in the radial axis (Fig. 2A). This growth profile can be considered as a 'standing wave' of growth (Silk and Erickson, 1978); the apical hook remains curved while the hypocotyl elongates. During hook opening, however, epidermal cell flow over the hook stopped (data not shown).

Detailed time-lapse photography of the strong *hls1-1* allele revealed a curved apical hypocotyl in seedlings which were 30-34 hours old (Fig. 2B) and epidermal cells 'flowed' over the hook midline (Fig. 2: 30-34). This observation indicates that this stage of hook formation in *hls1* seedlings started in the

normal way. However, 2 hours later the hook midline was shifted from its parallel position with the hypocotyl axis; cells stopped flowing over the apex while the hook structure started to open (Fig. 2: 36). These results suggest that HLS is required for early maintenance of differential growth in the apical hook.

Requirement of ethylene for hook development

Arabidopsis seedlings grown in ethylene for 3 days display exaggerated hook curvature (reviewed by Ecker, 1995). As the apical hook is a transient structure, it is expected that ethylene sensitivity in the apical hypocotyl would be time-dependent. To determine the interval during which the apical hook shows sensitivity to ethylene, etiolated seedlings were grown in air for various time periods and then treated with ethylene (Fig. 3A). Seedlings 48 hours after imbibition in ethylene exhibited reduced curvature when compared to air-grown seedlings. When grown in air for 24 hours and shifted to ethylene, 48-hour-old seedlings exhibited an air-like hook curvature. However, seedlings 60 hours old that were first grown in air and then treated with ethylene for 12 or 36 hours exhibited an exaggeration of hook curvature, similar to seedlings that were grown in ethylene for 60 hours. When 72-hour-old seedlings were transferred to ethylene the exaggerated hook was not formed, although limited ethylene responsiveness was

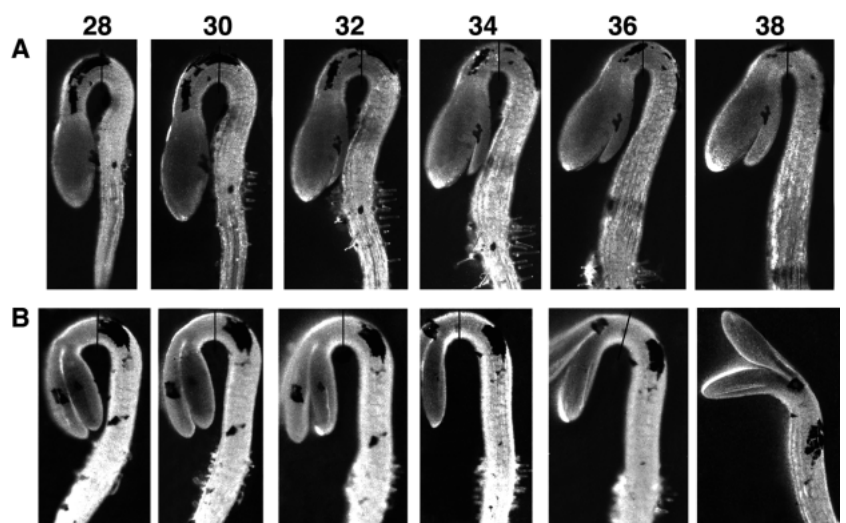


Fig. 2. Early steps in apical hook development in wild-type and *hls1* seedlings. Time-lapse photographs of Col wild-type (A) and *hls1-1* (B) seedlings germinated in air. Photographs were taken 28, 30, 32, 34, 36 and 38 hours after imbibing. Epidermal cells were marked at hour 28 with black ink. The hook midline is indicated by a straight line.

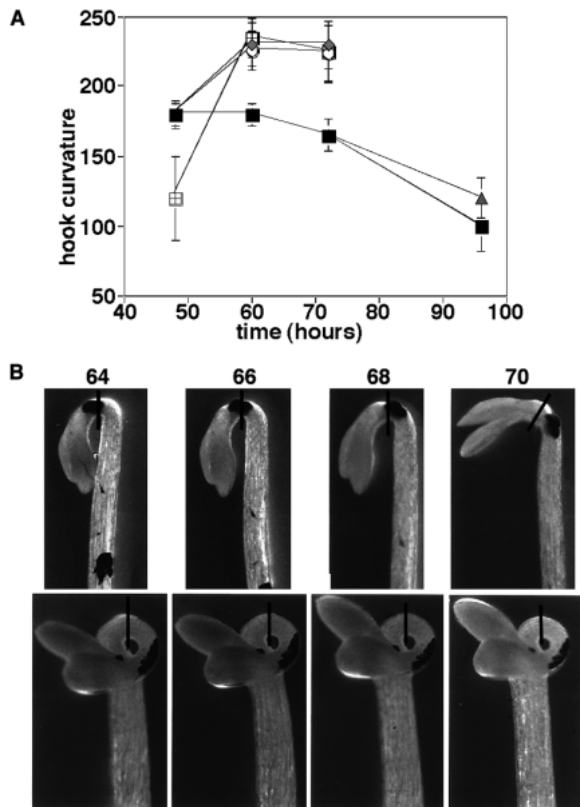


Fig. 3. Ethylene mediated hook development in etiolated seedlings. (A) Seedling growth conditions: 96 hours air (black squares), 24 hours air and 48 hours ethylene (diamond), 48 hours air and 48 hours ethylene (circle) or 72 hours air and 24 hours ethylene (triangle). As a control, seedlings were germinated in and grown in ethylene for 72 hours (squares with a cross). Hook curvature was measured at 12-hour intervals. Values are mean \pm s.d. of 30 seedlings. (B) Time-lapse photography of ethylene-induced apical hooks. Seeds were germinated in darkness in air (upper panel) or ethylene (lower panel); photographs were taken of 64-, 66-, 68- and 70-hour-old seedlings. During photography, the ethylene-grown seedlings were transferred to AT plates containing 10 μ M ACC. Epidermal cells were marked with black ink. The straight line indicates the hook mid-line.

sustained. These results indicate that apical hook sensitivity to ethylene is restricted to the phase between 60-72 hours. Before 60 hours, ethylene may have a negative effect on hook curvature. After 72 hours, ethylene sensitivity at the apical hook is reduced and the exaggerated structure does not develop. The formation of the exaggerated hook structure requires continuous exposure to ethylene, as seedlings grown in ethylene for 48 hours first and then after transferred to air for 12 hours displayed 'air-grown' hook curvature (Fig. 6).

In air-grown seedlings, the process of differential cell elongation maintains the curved structure of the apical hypocotyl. A difference between the growth rate of cells in both sides of the hook results in a curved structure (Silk and Erickson, 1978). Based on these observations the exaggerated curvature can be formed by a difference in the growth rate between the inner cells and the outer cells (i.e. cells at the inner side grow slower or cells at the outer side grow faster). Surprisingly, comparison between time-lapse photography of air-grown seedlings and ethylene-grown seedlings revealed

that ethylene induced a dramatic reduction in the growth rate of epidermal cells on both sides of the hook. The analysis of approximately 30 seedlings showed that marked cells along the hook at the outer and inner sides did not flow through the hook midline and their position relative to the hook midline or relative to other marked cells only slightly changed (Fig. 3B, low panel). In air-grown seedlings, however, cells flowed through the hook midline during the same time interval (Fig. 3B, upper panel). At 70 hours, the hook started to open in air-grown seedlings; an effect likely due to the accumulated amount of light during photography. The reduction in seedling growth rate was not restricted to the apical part of the hook, as cells at the basal part of the hook also stayed at the same position relative to the hook mid-line. As cells exhibited reduced elongation in response to ethylene, two possible mechanisms can be postulated to explain the role of ethylene in the formation of the exaggerated structure; only cells at the inner side of the hook respond to ethylene, cells at the outer side might respond to the inner cells with a reduced growth rate. Alternatively, cells on both sides of the hook may differentially respond to ethylene. As a result, growth rate inhibition of cells on each side of the hook is slightly different.

***ein*⁻ and *hls*⁻ mutants regulate separate steps during hook development**

As described above, time-dependent observations of seedling development suggest that several distinct factors control hook establishment and maintenance. To further examine this concept, hook curvature in *hookless* (*hls*) and *ethylene insensitive* (*ein*) mutants was measured during seedling growth, since the apical hook of both mutants are affected (Roman et al., 1995). Among the various *ein* mutants, we studied the ethylene-receptor mutants, *etr1* and *ein4* (Hua and Meyerowitz, 1998; Roman et al., 1995), and the downstream ethylene pathway mutants *ein2-5* and *ein3* (Roman et al., 1995, Chao et al., 1997). One-day-old etiolated mutant seedlings exhibited a variable hook curvature that was independent of genotype. During the second day of seedling growth, curvature of the apical hook in wild-type seedlings increased, and this increase was maintained during the third day. However, during the fourth day of growth hook curvature began to decrease (Fig. 4A). In the *ein* mutants, 2-day-old seedlings exhibited a curved hook similar to the wild type. However, the hook opened in three-day-old *ein* seedlings (Fig. 4A). In the weak *etr1* allele (*etr1-3*) the hook was partially open in 3-day-old seedlings. Differences in hook curvature between the *ein* mutants were measured in 3-day-old seedlings (Fig. 4B). These can be grouped in two phenotypic classes: *etr1-1* and *ein2*, in which the hook was opened by the third day, and *ein4* and *ein3* seedlings, in which the hook was only partly opened by the third day.

The profile of hook curvature in the *hls* mutants, *hls1-1* and *cop2*, was very different. The 'hook' of *hls* mutants was opened in 2-day-old seedlings (Fig. 4A); 1 day earlier than the hook of the *ein* mutants. This observation was further supported by analysis of double mutants. In 2-day-old seedlings, the hook was open in all combinations of *hls, ein* double mutants (Fig. 4B,C). The double mutants *cop2, etr1-1* and *hls1-1, etr1-1* exhibited *hls* morphology in the apical hook while the root and the hypocotyl were long and showed ethylene insensitivity (Fig. 4C). These results suggest that different mechanisms may

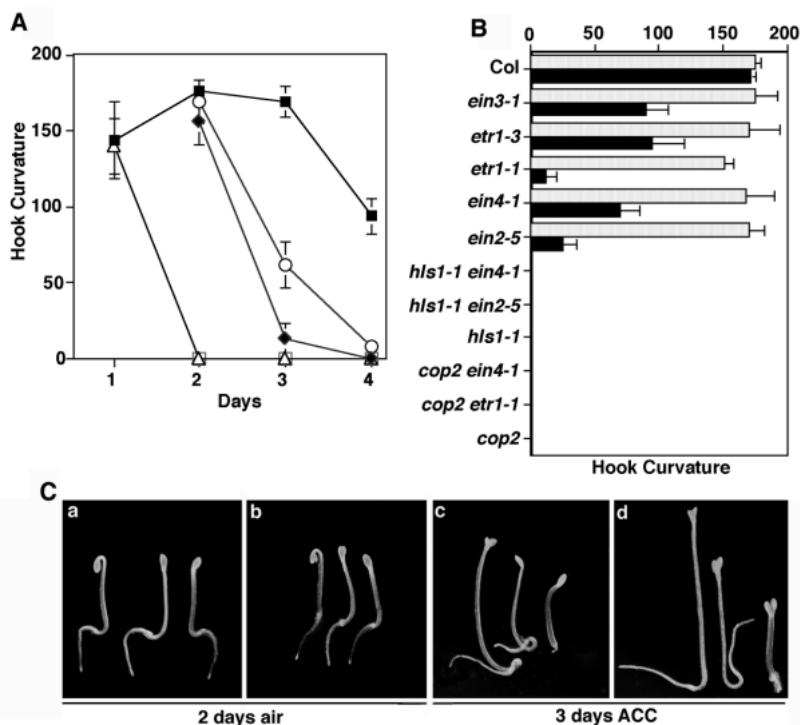


Fig. 4. Apical hook development in hook-affected mutants. (A) Development of the apical hook curvature of air-grown seedlings was measured over the first four days after imbibing: wild-type (black squares); *etr1-1* (black diamonds); *ein4-1* (white circles); *hls1-1* (white triangles); *cop2* (white squares). Differences in germination rates were normalized according to the root length. (B) Apical hook curvature of air-grown seedlings was measured in seedlings at 2 (dotted bars) and 3 (black bars) days of age. Values are mean \pm s.d. of 50 seedlings. Germination differences were normalized according to the root length. (C) Double mutant analyses. Photographs show 2-day-old seedlings grown in air (a,b); 3-day-old seedlings in 10 μ M ACC (c,d). Left to right in each panel: *etr1-1*, *hls1-1 etr1-1*, *hls1-1* (a,c); *etr1-1*, *cop2 etr1-1*, *cop2* (b,d).

act to control elongation in the apical hook and in the hypocotyl and the root.

Expression of *ETR1* and *AtACO2* in the apical hook

As cells on both sides of the apical hook have been observed to elongate at different rates, it might be expected that they may express different set of genes. To test this hypothesis, we selected for genes that are differentially expressed in the apical hook, focusing on ethylene-regulated genes. The expression of *ETR1* and *AtACO2* transcripts were analyzed using the whole-mount in situ hybridization technique. As a control for the technique, seedlings were hybridized with DIG-labeled oligo-dT, which revealed relatively uniform staining (relative to cell volume) in all hypocotyl cells (not shown). As a negative control, sense-labeled *ETR1* (Fig. 5Aa) and *AtACO2* (not shown) riboprobes were hybridized to the seedling preparations. A similar level of background staining was obtained in ethylene-grown *etr1-1* seedlings after hybridization with the anti-sense probes (Fig. 5Bc and 5Cc respectively). In the air-grown 2-day-old seedlings, *ETR1* mRNA showed uniform expression throughout the hypocotyl and the apical hook (Fig. 5Ab). By day 3, the transcript was predominantly enriched in the hypocotyl (Fig. 5Ba). In the presence of ethylene, its expression was further reduced in the apical part of the hypocotyl (Fig. 5Bb). These results suggest that the expression of *ETR1* in the apical hypocotyl is negatively regulated by ethylene. The reduced expression of *ETR1* in *etr1-1* seedlings (Fig. 5Bc) may suggest an auto-regulation of this gene.

AtACO2/EI305 expression is ethylene-dependent (Kieber et al., 1993 and Fig. 5C). In etiolated seedlings the transcript was not detected, using Northern blot analyses, in *ein* mutants grown in ethylene, nor in Col WT and in *hls* mutants grown in air (not shown). Sequencing of the *EI305* cDNA revealed that it encoded a new member of the ACC oxidase gene

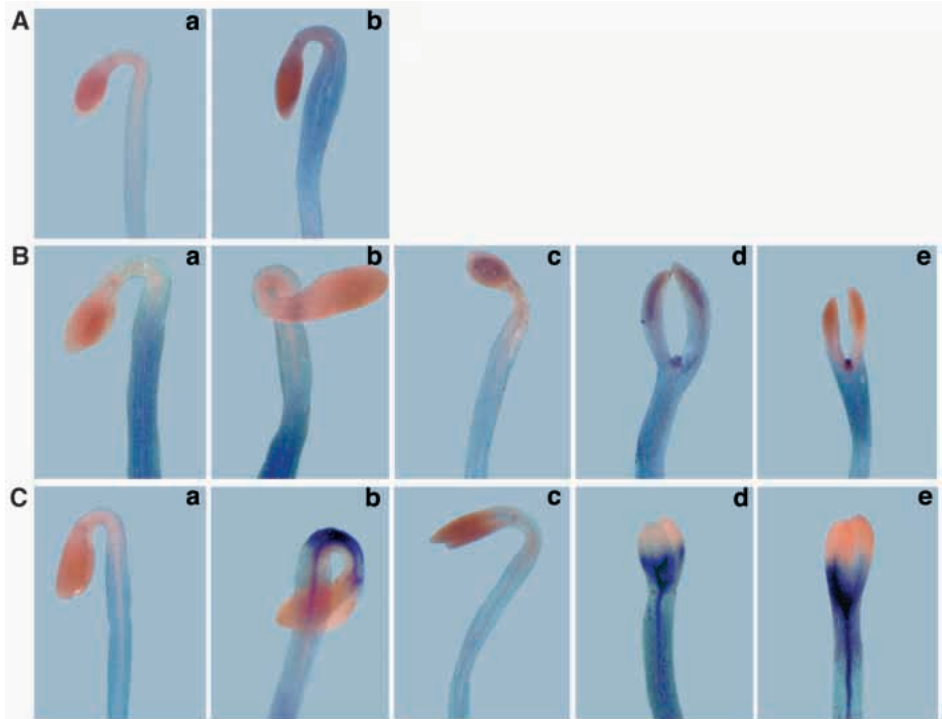
family (see Methods). ACC oxidase is the last enzyme in the ethylene biosynthetic pathway, and converts ACC to ethylene via oxidation of the amino group (reviewed by Ecker and Theologis, 1994). As *EI305* is the second ACC oxidase that has been isolated from *Arabidopsis* (Gomez-Lim et al., 1993), we named it *AtACO2*. The ACC oxidase gene family in *Arabidopsis* includes at least four members, as determined by Southern blot analysis (see methods). The *AtACO2* transcript was localized in cells at the outer side of the exaggerated hook in an ethylene-dependent manner (Fig. 5Ca,b).

The expression pattern of *AtACO2* and *ETR1* was also analyzed in ethylene grown *hls1-1* and *cop2* seedlings. Whereas *AtACO2* expression in the exaggerated hook of wild-type seedlings was differentially localized, in the 'hook-region' of *hls1-1* and *cop2* mutants it was expressed on both sides of the hypocotyl (Fig. 5Cd and Ce, respectively). The presence of *AtACO2* RNA in the apical hypocotyl of the *hls* mutants indicates that the ethylene signal was perceived and transduced, whereas the signal for ethylene-mediated differential growth was not. The expression of *ETR1* in ethylene-grown *hls* seedlings was expanded towards the apical part of the hypocotyl (Fig. 5Cd and 5Ce), while in wild-type seedlings *ETR1* expression was absent in the hook (Fig. 5Bb). These results suggest that *HLS* and *COP2* are necessary for spatial restriction of *ETR1* expression in the apical hook. In addition, the expression of *ETR1* and *AtACO2* in the *hls* apical hypocotyl suggest that ethylene perception and signal transduction in the apical hook of these mutants is at least partially intact.

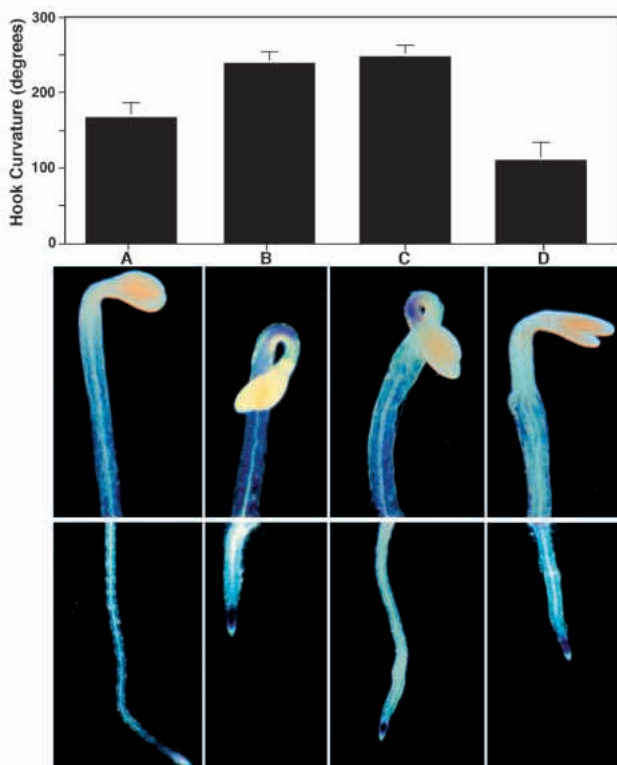
AtACO2 expression is a marker for ethylene sensitivity in the apical hook

As shown, *AtACO2* mRNA exhibited a highly localized expression pattern in the apical hook, so it could be used as a possible marker for ethylene sensitivity in that region. To

Fig. 5. Ethylene-regulated gene expression in the apical hypocotyl. Whole-mount in situ hybridization with DIG-labeled probes on etiolated seedlings. Hybridization is visible as blue/purple staining. (A) Air-grown 2-day-old seedlings hybridized with the sense (a), or antisense (b) *ETR1* probes. (B) Three-day-old seedlings hybridized with *ETR1* antisense probe: wild-type (a,b); *etr1-1* (c), *hls1-1* (d) and *cop2* (e) seedlings grown in air (a) or in ethylene (b-e). (C) Three-day-old seedlings hybridized with *AtACO2* antisense probe: wild-type (a,b); *etr1-1* (c), *hls1-1* (d) and *cop2* (e) seedlings grown in air (a) or in ethylene (b-e).



delimit the period of ethylene responsiveness during hook development, wild-type seedlings were used in ethylene-shift experiments. Seedlings were grown in air for 48 hours and were moved into an ethylene chamber for 20 hours (Fig. 6C). Reciprocally, seedlings were first grown in ethylene for 48 hours and then shifted into an air chamber for 20 hours (Fig.



6D). As controls, seedlings were grown in air or in ethylene for 3 days (Fig. 6A and B, respectively). Hook curvature was measured and the expression of *AtACO2* was examined using whole-mount in situ hybridization. *AtACO2* mRNA accumulation in the apical hook was found to be tightly correlated with formation of the exaggerated hook in both continuous growth in ethylene or air-ethylene shift experiments (Fig. 6C,B). When seedlings were grown under the reciprocal ethylene-air condition, *AtACO2* mRNA was not detected in the apical hook, and the exaggerated hook was not formed (Fig. 6D). These results reveal a transient sensitivity to ethylene in cells of the apical hook, and a positive correlation between hook curvature and *AtACO2* expression in the apical hook. *AtACO2* RNA was also detected in the root tip and showed ethylene dependency, whereas no staining was observed in air-grown seedlings (Fig. 6A). However, *AtACO2* expression in the root was not affected by the reciprocal air-ethylene and ethylene-air conditions (Fig. 6C,D). The difference in the regulation of *AtACO2* expression between the root tip and the apical hook may indicate the presence of two different tissue-specific ethylene response pathways. Alternatively, the threshold for ethylene sensitivity in these tissues might be different.

Fig. 6. Hook curvature and *AtACO2* expression in seedlings grown under ethylene-shift conditions. Wild-type seedlings were grown in a continuous flow of (A) air or (B) ethylene for 68 hours. (C) Seedlings were grown in air for 48 hours followed by ethylene for 20 hours (air-ethylene). In the ethylene-air treatment (D) seedlings were grown in ethylene for 48 hours followed by air for 20 hours. Hook curvature was measured and seedlings were subjected to the whole-mount in situ hybridization. Upper panel and middle panel show apical part and roots of seedlings hybridized with DIG-labeled *AtACO2* antisense riboprobe. Hybridization is visible as purple staining. Values are mean \pm s.d. of 50 seedlings.

DISCUSSION

The apical hook in *Arabidopsis* is a dynamic structure formed in dark-grown seedlings. However, this structure has mainly been studied using 3-day-old seedlings (Lehman et al., 1996 and references therein). Here we considered the apical hook as a developmental structure, which is transiently formed during early stages of seedling growth. We divide the process of apical hook development into three sequential phases (Fig. 7). The assignment of each phase is based on the position of the hook midline relative to the hypocotyl axis. During hook formation the hook midline is placed parallel to the apical-basal axis of the seedling while the hook curvature is increased. During the maintenance phase the hook midline maintains its parallel position with the hypocotyl axis as well as its curvature, while cells continue to flow through the hook apex in the apical-basal direction while rotating around the hypocotyl. Thus, hook maintenance is probably regulated by differential growth rate along the apical basal axis and in around the radial axis. Finally, the parallel position of the hook midline turns and the hook open. The distinct mechanisms of coordination of cell growth rates on each side of the hook during the three steps may suggest distinct modes of regulation.

The process of hook development is regulated by a variety of plant hormones including ethylene, auxin, cytokinin (Lehman et al., 1996) and presumably also by brassinosteroids as these hormones appear to play a role in de-etiolation (Li et al., 1996). Thus, many of the mutants that affect hormone metabolism or signaling may exhibit aberrant hook morphology. We studied the role of ethylene in hook development using the *ethylene-insensitive* mutants. Our studies revealed that the main role of ethylene is in hook maintenance, and cells in the hook are sensitive to ethylene only for a restricted time during seedling growth (Fig. 7). Furthermore, the time-dependent analyses of the hook show that the *ein* and *hls* mutants, which were thought to affect the same process, are affecting distinct steps during apical hook development (Fig. 7). Thus, the apical hook of *hls1* and *cop2* mutants do not curve in response to ethylene because the hook is misformed during an earlier developmental step. At the molecular level, however, both mutants show ethylene-responsiveness in the apical hook, as shown by the expression of *AtACO2* and *Etr1* transcripts. The expression of these genes is spatially abnormal since the structure is deformed. Developmental analysis of hook curvature during seedling growth can be applied to additional mutants involved in other hormone-signaling pathways. Such studies may allow us to better understand the role of hormones and the possible interactions between them during differential growth.

Differential growth of the hypocotyl also occurs in response to light or gravity. In these processes, the hypocotyl bends towards the stimulus in a process requiring differential cell elongation (Firn and Digby, 1980; Estelle, 1996). However, unlike differential growth in the apical hook where the process in *Arabidopsis* is irreversible, in tropic responses, the tissue shows sensitivity each time it is exposed to the stimuli. Thus, the regulation of differential growth in these cases may be distinct, but similar to hypocotyl re-etiolation in epigeal spices, such as pea. Indeed the *SAUR* gene, an auxin response gene (Gil et al., 1994), is differentially expressed in the hypocotyl during the tropism process (Li et al., 1991), whereas it is not

differentially expressed in the apical hook (Lehman et al., 1996). As ethylene is involved in the gravitropic response (Golan et al., 1996), it will be interesting to examine the expression pattern of *AtACO2* in the hypocotyl after gravistimulation.

Using molecular markers as tools to probe differential growth a finer resolution of hook development may be achieved. In this work we present such an example. The expression pattern of *AtACO2* and *ETR1* in the apical hook is affected by ethylene in complex ways. *ETR1* is expressed early in the apical hook during seedling growth, but once the structure becomes ethylene-sensitive its expression in the hook fades. These results suggest that the expression of *ETR1* in the apical hook may be negatively regulated by ethylene. The extended expression of *ETR1* in the apical hypocotyl of *hls1* and *cop2* suggests that *ETR1* expression in the apical hook is under the regulation of HLS genes. However *etr1-1* is a gain-of-function allele, the role of *ETR1* in differential growth in the apical hook remains to be analyzed in a loss-of-function allele (Hua and Meyerowitz, 1998). The expression of *AtACO2* in the apical hook, however, is positively regulated by ethylene and restricted to the time period that the cells are ethylene-sensitive. The *AtACO2* transcripts exhibited differential localization in the hook and more specifically, accumulated in the outer cells of the hook. This is consistent with the observation that ACC accumulates in cells of the outside part of the hook in *Phaseolus vulgaris* seedlings (Schwark and Bopp, 1993). Asymmetric localization of *PsACO1* transcripts in pea apical hook was recently reported (Perk et al., 1998). Unlike *AtACO2* localization in *Arabidopsis*, *PsACO1* RNA accumulated in the inner cells of the hook in air grown

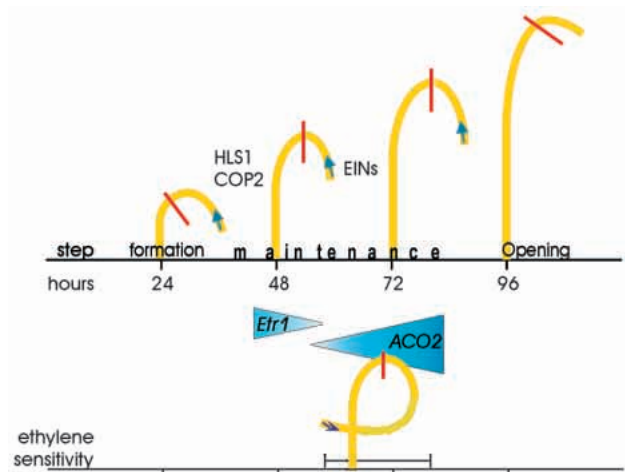


Fig. 7. A scheme for regulation of apical hook development during seedling growth. Early during seedling growth the hook is formed and displaced to the apical region of hypocotyl. Subsequently the apical hook is maintained by *HLS1* and *COP2*. These genes negatively regulate the expression of *ETR1* in the apical hook. Later, the structure becomes sensitive to ethylene and the *EIN* genes are required for maintenance of the hook. At this time, an increased ethylene level can induce the exaggerated hook morphology together with the expression of *AtACO2* in the apical hook. After 96 hours, maintenance of the hook discontinues, and the hook begins to open. Red lines mark the hook midline. Arrows show the direction of cell flow over the apical hook.

seedlings. *PsACO1* expression level was also elevated by ethylene and the transcript was expressed on both sides of the hook, although it was more abundant in cells on the inner side of the hook. The apical hook in pea and *Arabidopsis* are formed differently. In pea, an epigeal plant, the apical hook is placed above the cotyledons, while in *Arabidopsis*, a hypogeal plant, the apical hook is placed below the cotyledons. Ethylene regulation of apical hook development in epigeal and hypogeal plants may be different. ACC oxidases are encoded by a multi-gene family and developmental patterns of gene expression may respond to different regulation processes (Barry et al., 1996). The regulation of *AtACO2* and *PsACO1* is clearly different as only *PsACO1* is expressed in air. The distinct patterns of localization of these two ACO genes on opposite sides of the hook suggests that ethylene regulates distinct responses in the apical hook in these different species. Thus, it would be interesting to analyze the expression of other ACC oxidases in the apical hook.

Thus far, our efforts to identify and characterize molecular markers that are differentially expressed in the hook have been concentrated on ethylene-regulated genes. In light of the two distinct phases of hook development, the identification of additional genes, which are expressed early during seedling growth, should provide additional insight into the patterning of cells that undergo differential responses to hormone signals.

We thank M. Koornneef, S. de Vries and J. Weller for critical reading of the manuscript, and Christopher Kim and Ken Dewar for assisting in mapping the *AtACO2* gene. V. R. was a recipient of the Fulbright Fellowship. This work was supported by a grant from the Department of Energy (DE-FG-02-93ER20104).

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