FINAL TECHNICAL REPORT

DE-FG02-93ER20104  P. I. JOSEPH R. ECKER- UNIVERSITY OF PENNSYLVANIA

1. Period of support: 5/1/93- 4/30/00

2. SUMMARY STATEMENT

3. Project Title: Molecular and Genetic Analysis of Hormone-Regulated Differential Cell Elongation in Arabidopsis

4. Executive Summary: We have utilized the response of Arabidopsis seedlings to the plant hormone ethylene to identify new genes involved in the regulation of ethylene biosynthesis, perception, signal transduction and differential cell growth. In building a genetic framework for the action of these genes, we developed a molecular model that has facilitated our understanding of the molecular requirements of ethylene for cell elongation processes. The ethylene response pathway in Arabidopsis appears to be primarily linear and is defined by the genes: ETR1, ETR2, ERS1, ERS2, EIN4, CTR1, EIN2, EIN3, EIN5, EIN6, and EIN. Downstream branches identified by the HLS1, EIR1, and AUX1 genes involve interactions with other hormonal (auxin) signals in the process of differential cell elongation in the hypocotyl hook. Cloning and characterization of HLS1 and three HLS1-LIKE genes in my laboratory has been supported under this award. HLS1 is required for differential elongation of cells in the hypocotyl and may act in the establishment of hormone gradients. Also during the award period, we have identified and begun preliminary characterization of two genes that genetically act upstream of the ethylene receptors. ETI1 and RAN1 encode negative regulators of ethylene biosynthesis and signaling respectively. Progress on the analysis of these genes along with HOOKLESS1 is described.

5. Human Resource Development: Participants supported under in this DOE-funded project have included: four postdoctoral fellows: Claire Lurin, Kevin Wang, Jose M. Alonso, Vered Raz, Hitoshi Yoshida, Takashi Hirayama and one graduate student: Anne Lehman. Numerous undergraduates have also been trained as part of this research program.

6. Publications: (5/94 – 4/00)


Yoshida, H., Wang, L.C., Lurin, C. and Ecker, J.R. ETI1, a Negative Regulator of Ethylene Biosynthesis, Encodes an Novel ACC Synthase-Interacting TPR-Protein, manuscript in preparation

Other publications*

DOE Patent Clearance Granted

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7. DESCRIPTION OF COMPLETED STUDIES

A) SUMMARY OF SIGNIFICANT PUBLICATIONS


(abstract) Bending in plant tissues results from differential cell elongation. We have characterized Arabidopsis "hookless" mutants that are defective in differential growth in the hypocotyl. HOOKLESS was cloned and its predicted protein shows similarity to a diverse group of N-acetyltransferases. HOOKLESS mRNA is increased by treatment with ethylene and decreased in the ethylene insensitive mutant ein2. High level expression of HOOKLESS mRNA results in constitutive hook curvature. The morphology of the "hookless" hypocotyl is phenocopied by inhibitors of auxin transport or by high levels of endogenous or exogenous auxin. Spatial patterns of expression of two immediate early, auxin-responsive genes are altered in hookless mutants, suggesting that the ethylene response gene HOOKLESS controls differential cell growth by regulating auxin activity.

2. RESPONSIVE-TO-ANTAGONISTI, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis (Hirayama, T., Kieber, J., Hirayama, N., Kogan, M., Guzman, F., Nourizadeh, S., Alonso, J.M., Dailey, W.P., Dancis, A. and Ecker, J.R. 1999 Cell, 97:382-393) (Abstract) Ethylene is an important regulator of plant growth. We identified an Arabidopsis mutant, responsive-to-antagonist 1 (ran1), that shows ethylene phenotypes in response to treatment with trans-cyclooctene, a potent receptor antagonist. Genetic epistasis studies revealed an early requirement for RAN1 in the ethylene pathway. RAN1 was cloned and found to encode a protein with similarity to copper transporting P-type ATPases including the human Menkes/Wilson proteins and yeast Ccc2p. Expression of RAN1 complemented the defects of a ccc2Δ mutant, demonstrating its function as a copper transporter. Transgenic CaMV 35S::RAN1 plants showed constitutive expression of ethylene responses, due to cosuppression of RAN1. These results provide the first in planta demonstration that ethylene signaling requires copper and reveal that RAN1 acts by delivering copper to create functional

ABSTRACT: Ethylene regulates plant growth, development and responsiveness to a variety of stresses. Cloning of the Arabidopsis EIN2 gene identifies a central component of the ethylene-signaling pathway. The amino-terminal integral membrane domain of EIN2 shows similarity to the disease-related NAMP family of metal ion transporters. Expression of the EIN2 CEND is sufficient to constitutively activate ethylene responses, and restores responsiveness to jasmonic acid and paraquat-induced oxygen radicals to mutant plants. EIN2 is thus recognized as a molecular link between previously distinct hormone response pathways. Plants may use a combinatorial mechanism for assessing various stresses by employing a common set of signaling molecules.

4. Regulation of differential growth in the apical hook of Arabidopsis. (Raz and Ecker, 1999 Development, in press) ABSTRACT: 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 (hsl1, 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.

5. ETO1, a Negative Regulator of Ethylene Biosynthesis, Encodes a Novel ACC Synthase Interacting TPR-Domain Protein, (Yoshida, H., Wang, L.C., Lurin, C. and Ecker, J.R. Manuscript nearing submission) Characterization of the ETO1 has revealed that this gene encodes a novel plant protein with tetratricopeptide repeat (TPR) motifs in its C-terminus. TPR motif is a degenerated 34 amino acid sequence identified in a variety of proteins and proposed to be involved in protein-protein interaction suggesting the action of ETO1 through protein-protein interaction. Putative homologs of ETO1 were also identified and include, the ETO1-LIKE genes from Arabidopsis (EOL1 and EOL2), rice (PEO), poplar (PEO) and other plant species. These predicted proteins are highly similar to each other and contain TPR motifs in the C-terminus, suggesting their functional similarity and implying a common system for regulating the biosynthesis of ethylene in a broad cross-section of the plant kingdom. Mutations in the TPR domain of the ETO1 resulted in overproduction of ethylene, suggesting that this protein-protein interaction domain is required for the negative regulation of the ethylene biosynthesis.

B) SUMMARY OF UNPUBLISHED RESULTS

The role of HOOKLESS1 and HOOKLESS1-LIKE genes in differential growth
In addition to the above published study (Raz and Ecker 1999, Development, in press), we have taken several approaches in the past grant period (3.2 years) to gain further insights into the role of HOOKLESS1 plays in hormone-mediated growth processes. These include 1) Isolation and characterization of genetic suppressors of hsl1-1, 2) Identification of HLS1-interacting proteins and 3) Isolation, and characterization of mutations in HLS1-LIKE genes (HLL1, HLL2, and HLL3).

1. Isolation and characterization of hookless1 extragenic suppressors. The identification of
suppressors of HLS1 could potentially yield proteins that interact with HLS1 or genes that act in the same pathway that regulates differential growth in the apical hook. The hls1-I mutant was chosen because it encodes a missense mutation that changes Glu 346 to Lys and it is a strong allele.

a) Identification of hookless1 extragenic suppressors (hss mutants). Seeds were mutagenized by both EMS and fast-neutron treatment. Approximately 60,000 freshly harvested hls1-I seeds (1.2g) were treated EMS and dispersed into 20 pools (2k/pool). The plants were grown in the greenhouse for 2 months. A total of 114.5 g of seed was harvested. For screening, 40-45 mgs of seed (~2000 seeds) were resuspended in AT top agar, plated on large AT plates containing 10 uM ACC, exposed to light for 2-4 hours, then wrapped in foil and placed in the cold for 4 days. They were moved to 24C for 72 hours, then screened. 293 M2 seedlings were picked and 71 plant survived to maturation and produced seed. The M3 plants were rescreened for suppression of hls1-I phenotypes throughout development. A line was considered to suppress the hls1-I defect if the cotyledons remained closed and the petals were shorter than hls1-I at the 3-day etiolated stage. Suppressors also bolted later (by ~1 week) than hls1-I, and they produced one sturdy bolt, rather than multiple bolts that were thinner than their wild-type counterparts. The suppressors had larger rosettes than hls1-I plants and they senesced later than hls1-I plants. The senescence phenotype might be the result of a sterility defect. Only late (terminal) flowers of the suppressor lines produced seed. Earlier flowers contained immature anthers that lacked pollen grains, but the gynoecium of these flowers was fertile, as it was receptive to crosses.

Two separate screens of 40,000 seedlings each yielded 3 lines (1-3, 4-7, and 15-1) that re-tested as partial suppressors which were called hss (hookless1 suppressor) mutants. Fast-neutron mutagenized seed was obtained by mailing freshly harvested hls1-I seed to Paolo Donini at the International Atomic Energy Agency in Vienna. After 60 Gy treatment of fast neutron radiation, 0.73g of seed were resuspended in 0.1% agarose and distributed over 30 flats of moistened Metromix and grown in a 24° growth chamber. Screening of 60,000 seedlings yielded complete suppressors in 3 different pools (3-9 seedlings per pool). To determine whether the suppressors contained the hls1-I mutation, primers were designed that created a BslI site when they were used to amplify a PCR fragment from genomic DNA isolated from the lines in question. Amplification of a 159 bp fragment, followed by digestion with BstII, yielded 2 fragments in wt plants of 145 and 14 bp, whereas fragments amplified from hls1-I DNA can not be cut. A line heterozygous for the hls1-I mutation has both the 159 bp and 145 bp bands, which can be distinguished on a 3.7% agarose gel.

b) Characterization of the hss mutants. A number of crosses were performed to elucidate the nature of the suppressor mutation. A cross of hss1-3 and hss4-7 to hls1-I gave rise to F1 plants that had none of the characteristics of the suppressors and resembled the hls1-I parent; therefore, the hss mutation is recessive. The suppressor phenotype reappeared in the F2. A cross of hss1-3 and hss4-7 to Columbia was performed to determine whether the suppressor mutation had a phenotype on its own. As expected for these recessive mutations, the F1 plants were wild type. In the F2, there was a mixture of wild-type, hls1-I and suppressed plants. Scoring of each class was not determined because the phenotype was difficult to accurately determine at the seedling stage. Crosses of the suppressors to hls1-I (another strong missense allele) a hls1-I mutant in the Ler background were performed to determine allele specificity. All crosses revealed that the suppressor mutations are not allele-specific. In the case of the crosses to Ler hls1, the ratios of hls:suppressor phenotypes in the F2 were determined, and the suppressed plants were used for mapping (see below). It was necessary to grow plants to adulthood in order to score phenotypes accurately, in particular, the late senescence and sterile phenotypes. 206 F2 plants from the hss1-3 x Ler hls1 cross gave 164:42 hls:suppressor, or 3.9:1. F2 plants (105) from the hss4-7 x Ler hls1 cross produced 78:27 hls:suppressor, or 2.9:1. If the mutations were allele-specific, the expected ratio would be 15:1. Crosses of the hss1-3 and hss4-7 suppressors to each other (using pollen from terminal flowers) revealed that the mutants are allelic, because 5/6 seedlings from the cross displayed the suppressed phenotype at the 3-day etiolated stage and through adulthood. One of these plants was crossed to hss15-I, and the F1 progeny of that cross also displayed the suppressed phenotype, indicating that all 3 hss1 mutants are allelic. Note that the suppressed phenotype is not a complete rescue of the hls1-I defect.
c) Mapping the hsl1 mutation To determine an approximate map position of the hsl1 mutation, DNA from 26 plants was used to amplify SSLP markers. SSLP marker nGA280 (bottom chr. I), nga172 (top chr. III) and nga1126 (middle chr. II) were all unlinked. AthBIO2 (chr. II) in contrast, showed linkage to the hsl1-3 mutation. Suppressed F2 plants from the hsl4-7 × Ler hsl1 cross also showed linkage to AthBIO2, supporting the earlier result that hsl1-3 and hsl4-7 mutations are allelic. To map the hsl1-3 mutation more precisely, additional markers in the region were tested. nga168 lies to the south of AthBIO2 about 11 cm away. This marker was unlinked to the hsl mutations (26:45:21 Col-0; Ler). SSLP nGA361 lies ~14 cm to the north of AthBIO2 and is weakly linked (39:23:18). Marker ve017 also lies to the north of AthBIO2 and appears to be unlinked (32:79:19). In total, AthBIO2 segregated: 105 Col:17 het:4 Ler in this cross. This region of chromosome 2 has been completely sequenced by TIGR. New genetic markers to the north of AthBIO2 will be identified from the genomic sequence in order to further delimit the region containing HSS1. Using an expanded population of F2 recombinant plants, it should be possible to identify the HSS1 gene within the next 6 months.

2. Two-hybrid screen for HLS1-interacting proteins. HLS1 is an excellent candidate for 2-hybrid screening relative to some other substrates. There is now an abundant physical data emerging about the conserved active site domain. Crystallographic analysis of the catalytic domain of two types of acetyltransferases—one that modifies histones and another that modifies small molecules—have recently been solved. In both cases, the substrate-binding pocket lies adjacent to the acetyl-CoA binding domain. Initial attempts to use the full-length HLS1 were unsuccessful because the full-length protein is toxic when expressed in yeast. Therefore, screening for interacting proteins using only the N-terminal acetyltransferase substrate-binding domain of HLS1 might yield physiological relevant-interacting proteins.

a) Yeast two-hybrid plasmid constructs. Strains and plasmids were all obtained from Erica Golemis lab (Fox Chase Cancer Center Philadelphia). Fragments of the HLS1 gene (either the N-terminal acetyltransferase domain or the C-terminal unique portion) were amplified from the 43.11 cDNA (Lehman et al. 1996) using primers containing using restriction sites. The fragments were subcloned into the Invitrogen pCR2 vector, then the fragments were cut out and subcloned into pMW103 (LexA bait fusion) or pGKS8-1 (a lambda cl plasmid).

b) Screen for HLS1-interacting proteins. A screen for proteins that interact with the HLS1 C-terminal domain-LexA fusion of 150-200,000 transformants resulted in ~120 putative interacting clones. The Arabidopsis cDNAs that encode the library of interacting proteins are under control of a galactose-inducible promoter. All but three of the interacting clones required galactose for the interaction. The second reporter, LacZ, also is induced to varying degrees in multiple isolates, and only in the presence of galactose. Plasmids (30) containing putative interacting clones were isolated from these strains and will be further characterized.

3. Identification of mutations in the HLS1 homologs: HLL1, HLL2 and HLL3. We are developing a large collection of single T-DNA transformed lines containing mutations in a variety of hormone signaling pathway genes. Approximately 150,000 T-DNA insertion line were created and over 60,000 T1 lines have been grown and the seeds from individual lines collected (in collaboration between my group and Bill Crosby's group at the NRC-Canada). We have already deposited over 20,000 T-DNA lines in the Arabidopsis Biological Resource Center at Ohio State Univ. By the end of 1999, this number will increase to 100,000 lines. Three-dimensional pools of 10,000 lines have been created and each 10K block is screened for gene insertions using PCR amplification: 1 primer complementary the gene of interest and 1 primer complementary to the T-DNA border sequence. Using only 40 PCR reactions, a single plant line with T-DNA insertion can be identified in each 10K block. Screening of the first 10,000 lines for mutations in HLS1, HLS2 and HLS3 gene has yielded a confirmed "hit" in each of the these genes. The development of these materials has allowed the identification a growing list of mutations in plant signaling pathway (and other) genes.
B) ETHYLENE-OVERPRODUCER1 (ETO1) and ETHYLENE-LIKE (EOL) proteins in the regulation of ethylene biosynthesis.

The ethylene-overproducing mutants (eto) provide useful tools for identification of the still unknown regulating components in the ethylene biosynthesis pathway (Guzman and Ecker, 1990). eto mutants constitutively show the triple response phenotype in the absence of exogenously applied ethylene. eto mutants can be distinguished from etr mutants that also display the triple response in the absence of the hormone, because their phenotypes are suppressed by antagonists of ethylene biosynthesis or action. From these results eto mutants have been suggested to be impaired in biosynthetic or regulatory components of ethylene biosynthesis (Guzman and Ecker, 1990). eto1 is a recessive mutation that results in approximately 10-fold of ethylene-overproduction compared to wild type plant (Guzman and Ecker 1990), while eto2 and eto3 are dominant mutation that cause 20- and 100-fold increase of ethylene biosynthesis, respectively (Kieber et al. 1992). eto1 and eto3 mutants have been suggested to play a role as a posttranscriptional regulator of ACS (Woeste et al., 1999).

Cloning and characterization of the ETO1 and ETO1-LIKE genes. In order to explore the mechanisms of regulation of ethylene biosynthesis further, ETO1 was isolated by positional cloning (Yoshida et al., in preparation). In brief, the ETO1 gene was genetically mapped on the bottom of chromosome 3 using visible markers (Kounan et al., 1995). We fine-mapped eto1-4 mutation using simple sequence length polymorphism (SSLP) markers (Bell and Ecker, 1994) and cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993). By analysis of 1824 recombinant chromosomes derived from a mapping cross between eto1-4 and wild-type Landsberg erecta, we delimited the region of the chromosome containing ETO1 to a 60 kb region flanked by an SSLP and a CAPS marker. We examined length polymorphisms in this region using ten alleles of eto1, and found a polymorphism in one allele, eto1-3 (Figure 2A). Although this mutation was found within the predicted first intron, the nature of the mutation suggests that inefficient splicing would occur resulting in a truncated form of the protein. The DNA sequences of each of the mutant alleles of the ETO1 gene were determined by genomic sequencing using templates from four independent PCR reactions. Sequencing of 7 eto1 alleles, including X-ray, EMS and DEB-induced alleles, enabled the identification single base-pair changes in each of these genes, which result in nonsense or missense mutations. From this analysis we concluded this gene encodes ETO1. The predicted ETO1 protein does not contain a consensus sequence for any of the known protein targeting domains, or any predicted transmembrane domains. With the notable exception of two highly similar ETO1-LIKE (EOL1 and EOL2) genes, searches of the sequence databases revealed that ETO1 is a novel protein. Further analysis revealed that ETO1 EOL1, and EOL2 are tetraelectropeptide repeat (TPR) proteins, containing several complete and incomplete TPR motifs in their carboxyl terminal half. The TPR motif is a highly degenerate 34 amino acid sequence that is known to be involved in mediating protein-protein interactions (Das et al. 1998). This domain is found in proteins with diverse functions, including mitosis, transcription, splicing, protein transport, neurogenesis or molecular chaperone (see Das et al., 1998 and references therein). Support for the role of these TPR domains in ETO1 function was provided by the sequences of two eto1 alleles. eto1-1, an EMS-induced mutation, contained a stop codon resulting in truncation of only two TPR motifs while in DEB-induced eto1-5, a missense mutation was identified in one of the incomplete TPR motifs, implying an important role for this domain in the function of ETO. Furthermore, analysis of the complete sequences of seven of the ten eto1 alleles revealed that each of these genes contained mutations that are predicted to cause an alteration in, or truncation of, the TPR domain. These results strongly indicate that the TPR domain plays an indispensable role in the (unknown) function of the ETO1/EOL proteins.

Demonstration of direct interaction of ACS and ETO1/EOL proteins. The dominant eto2 mutation was found to lie in the ACS5 gene (Vogel et al.1998). This DEB induced mutation resulted from a one base insertion (a frame-shift mutation) causing an alteration in the C-terminal region of ACS5 gene. By an unexplained mechanism, truncation of carboxyl-terminus of ACS results in ethylene overproduction, suggesting the involvement of C-terminus of ACS in regulation of this enzyme (Vogel et al., 1998, and see PROPOSAL). An earlier report also indicated a possible role for the carboxyl-end of a tomato ACS in the regulation of enzyme activity (Li and Mattoo, 1994). ETO1 a negative regulator of ethylene