Anaerobiosis and Plant Growth Hormones Induce Two Genes Encoding 1-Aminocyclopropane-1-Carboxylate Synthase in Rice (*Oryza sativa* L.)

Thomas I. Zarembinski and Athanasios Theologis

Plant Gene Expression Center, Albany, California 94710

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The plant hormone ethylene is believed to be responsible for the ability of rice to grow in the deepwater regions of Southeast Asia. Ethylene production is induced by hypoxia, which is caused by flooding, because of enhanced activity of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, the key enzyme in the ethylene biosynthetic pathway. We have cloned three divergent members, (*OS-ACS1*, *OS-ACS2*, and *OS-ACS3*), of a multigene family encoding ACC synthase in rice. *OS-ACS1* resides on chromosome 3 and *OS-ACS3* on chromosome 5 in the rice genome. The *OS-ACS1* and *OS-ACS3* genes are induced by anaerobiosis and indoleacetic acid (IAA) + benzyladenine (BA) + LiCl treatment. The anaerobic induction is differential and tissue specific; *OS-ACS1* is induced in the shoots, whereas *OS-ACS3* is induced in the roots. These inductions are insensitive to protein synthesis inhibitors, suggesting that they are primary responses to the inducers. All three genes are actually induced when protein synthesis is inhibited, indicating that they may be under negative control or that their mRNAs are unstable. The *OS-ACS1* gene was structurally characterized, and the function of its encoded protein ($M_r = 53\ 112$ Da, pI 8.2) was confirmed by expression experiments in *Escherichia coli*. The protein contains all eleven invariant amino acid residues that are conserved between aminotransferases and ACC synthases cloned from various dicotyledonous plants. The amino acid sequence shares significant identity to other ACC synthases (69–34%) and is more similar to sequences in other plant species (69% with the tomato *LE-ACS3*) than to other rice ACC synthases (50–44%). The data suggest that the extraordinary degree of divergence among ACC synthase isoenzymes within each species arose early in plant evolution and before the divergence of monocotyledonous and dicotyledonous plants.

INTRODUCTION

When the roots of higher plants become anoxic under conditions of excessive rainfall, poor soil drainage, or frequent flooding, root growth ceases and eventually the roots die (Hook and Scholters, 1978; Roberts *et al.*, 1984; Jackson, 1985; Kennedy *et al.*, 1992). Only if the root tissue receives oxygen from aerobic plant tissues can root growth continue (Jackson, 1985; Hook and Scholters, 1978). Many dryland species have developed physiological adaptations to avoid root anoxia. For example, under flooded conditions, they develop aerenchyma, adventitious roots, and proliferate their lenticels (Jackson, 1985; Meent and Stolzy, 1978). A remarkable adaptation of higher plants to oxygen deficiency is found in rice and, in particular, in the deepwater varieties.

Deepwater rice responds to partial submergence with an enhancement in internodal growth (Jackson, 1985; Kende, 1987). Its survival depends on its ability to keep part of its foliage above water. Total submergence is deleterious to rice because the parts of the leaves above water facilitate the flow of air to the submerged parts of the plant to avoid anoxia (Raskin and Kende, 1985).

Ethylene is the key regulator of hypoxia-induced adaptations (Drew *et al.*, 1979; Yang and Hoffman, 1984; Jackson, 1985; Kende, 1987; Abeles *et al.*, 1992). In rice, ethylene production is greatly enhanced by hypoxia because of the induction of ACC¹ synthase (S-adenosyl-

¹ Abbreviations used: ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC synthase; Adh, alcohol dehydrogenase; AdoMet, S-ad-

L-methionine methythioadenosine-lyase, EC 4.4.1.14), the key regulatory step in the ethylene biosynthetic pathway (Yang and Hoffman, 1984; Cohen and Kende, 1987; Kende, 1987). The enhanced ethylene production is thought to be responsible for the increase in internodal length (Kende, 1987). In dicotyledonous plants, ethylene production is induced by plant growth hormones, including auxin and BA, as well as by the monovalent cation Li^+ (Yang and Hoffman, 1984; Boller, 1984). This induction is because of an increase in the ACC synthase mRNA (Sato and Theologis, 1989; Huang *et al.*, 1991; Nakagawa *et al.*, 1991; Sato *et al.*, 1991; Kim *et al.*, 1992; Liang *et al.*, 1992; Yip *et al.*, 1992). The role of these modulators of ethylene production in monocots (e.g., rice) remains unexplored.

We are interested in understanding the molecular basis of the regulation of ethylene production during hypoxia. Our long term goal is to demonstrate that ethylene is indeed the hormone responsible for growth of rice under submergence. Furthermore, understanding the molecular details of the signal transduction pathway(s) responsible for ethylene production may ultimately allow one to engineer high-yielding varieties for growth in deepwater areas (Kende, 1987). As a first step towards this goal, we have isolated three members of a multigene family in rice encoding ACC synthase and have investigated their expression characteristics in response to anaerobiosis, various hormones, and Li^+ ions. The structure of one of the genes, *OS-ACS1*, which is induced by anaerobiosis is described.

MATERIALS AND METHODS

Plant Material and Tissue Treatment

Rice seeds (*O. sativa* L. cv. IR36) were surface sterilized in 0.5% (wt/vol) sodium hypochlorite solution, and eight-day-old etiolated rice seedlings were grown on wet sterile sponge paper at 25°C, in the dark (Satler and Kende, 1985). For hormonal inductions, rice shoots were collected and incubated at 25°C in "incubation buffer" (100 mM KPO_4 , pH 6.8–50 μg chloramphenicol per ml) with or without IAA (0.5 mM), BA (0.1 mM), LiCl (50 mM), and cycloheximide (20 μM). The tissue was gently swirled for 40 min at 25°C, in the dark. The buffer was removed, and the tissue was incubated for 18 h at 25°C, in the dark. The tissue was frozen with liquid N_2 and stored at -80°C . The tissue that was immediately cut and frozen or treated with or without inducers was termed "intact", "induced", and "uninduced", respectively.

For the first set of anaerobic experiments (Figure 2, middle, and Figure 3), intact seedlings were treated with water-saturated air or N_2 for 12 h, in the dark. For the second set of experiments (Figure 2, right), the seedlings were first incubated in "incubation buffer" with or without cycloheximide (CHX) as described above and then treated with air or N_2 .

enosyl-methionine; bp, base pair; CHX, cycloheximide; IPCR, inverse polymerase chain reaction; nt, nucleotide; *OS*, *Oryza sativa*; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

PCR

The sequence and orientation of the degenerate primers TZ-1F and TZ-2R shown in Figure 1, panel I are as follows (see Figure 4 for their location):

TZ-1F $^{\alpha}$: 5'-GCC GAA TTC ATG GGN CTN GCN GAR AAY CA-3' (F) (324-343)

TZ-1F $^{\beta}$: 5'-GCC GAA TTC ATG GGN TTR GCN GAR AAY CA-3' (F) (324-343)

TZ-2R: 5'-GCC AAG CTT CCR TGR TAR TCY TGR AA-3' (R) (542-558)

N = A, C, G, or T; R = A or G; Y = T or C

TZ-1F is composed of TZ-1F $^{\alpha}$ and TZ-1F $^{\beta}$ in a 1:1 molar ratio; both encode the same primary sequences but differ at the degenerate codon combinations encoding leucine (Maniatis, *et al.*, 1989).

The gene-specific primers for the *OS-ACS1* gene are as follows: (see Figure 4 for their location):

A5a: 5'-GCC GAA TTC GAT GGT GAG CCA AGT GGT CG-3' (F) (167-186)

A11: 5'-GTA TAG CTC GAA GAA CAC TG-3' (R) (64-83)

B11: 5'-GCC AAG CTT CTA GTA TAG TGT ACT GTG AC-3' (R) (1992-2011)

B12: 5'-GCC AAG CTT TGT GGT TTT GTT CTT GGC GA-3' (R) (1923-1942)

A10a1: 5'-GCC GAA TTC GCG ACC ACT TGG CTC ACC AT-3' (R) (168-187)

B51: 5'-GCC AAG CTT CCG CGA GCC CGG CTG GTT CC-3' (F) (1697-1716)

Degenerate polymerase chain reactions (PCR) using the TZ-1F (512-fold degeneracy) and TZ-2R (32-fold degeneracy) primers and rice genomic DNA were carried out as described (Compton, 1990). The reaction mixture was incubated in a thermocycler (Perkin-Elmer-Cetus, Norwalk, CT) for 35 cycles: 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C. The PCR fragments were purified twice by gel electrophoresis and adsorption to Schleicher and Schuell (Keene, NH) NA-45 paper, followed by PCR reamplification using the same set of primers to enrich the amplified DNA fragments for subsequent subcloning and labeling. The PCR fragments were subcloned, sequenced, and termed pFOS-1, 2, and 3. The cDNA, prACS1, was synthesized as follows: first strand cDNA was synthesized by reverse transcription of mRNA from rice shoots treated with IAA+BzIAde+LiCl for 18 hr using B11 as primer, followed by PCR with primers A5a and B12 (Frohman *et al.*, 1988) in the presence of 150 μM 7-deaza-2'dGTP. The annealing temperature was 72°C. The PCR fragment was reamplified with A5a and B12 primers in the presence of 20% glycerol and 200 μM dGTP. The annealing and extension temperatures were 60°C (Henry and Oono, 1991). An inverse PCR product was synthesized with a circularized cDNA template and A10a1/B51 primers (Huang *et al.*, 1990). The product was subcloned into the *EcoRI*/*HindIII* sites of M13mp18 and 19 and was sequenced.

Construction of Genomic Libraries and Isolation of Genomic Clones

Genomic DNA was isolated from nuclei of etiolated seedlings by equilibrium sedimentation in CsCl gradients (Davis *et al.*, 1980; Del-

laporta *et al.*, 1983). Fragments (15–20 kb) from a partial *Sau*3A digest were cloned into the *Bam*HI site of λ DASHII (Stratagene, La Jolla, CA). The library was plated on *Escherichia coli* strain SURE (Stratagene). Five genomic equivalents (10^5 pfu) were screened with the use of 32 P-labeled pfOS-1,2,3 PCR inserts (Davis *et al.*, 1980; Rottmann *et al.*, 1991).

DNA Hybridization Analysis

Appropriate enzyme digests of phage DNAs or total genomic DNA were electrophoresed on 0.8% agarose gels, transferred to Nytran nylon membrane (Schleicher and Schuell) and hybridized with 32 P-labeled dsDNA inserts according to the manufacturer's instructions.

DNA Sequencing

The OS-ACS1 gene was sequenced with the use of the 4.65 kb *Eco*RI fragment from the genomic clone λ 10B1 (Fig. 1, II). The fragment was digested with *Sal* I to yield a 1.5-kb *Eco*RI/*Sal* I, a 0.66-kb *Sal* I, and a 3.5-kb *Sal* I/*Eco*RI fragments which were subcloned into pUC18. Both strands of OS-ACS1, prACS1, IPCR product, pfOS-2, and pfOS-3 were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) with Sequenase (United States Biochemicals, Cleveland, OH). Each DNA fragment was subcloned into M13mp18 and 19, and M13 universal primer, and synthetic primers (20mers) were used to sequence the ssDNAs (Sanger *et al.*, 1977; Messing, 1983). Inosine (United States Biochemical) or 7-deaza-2'-deoxyguanosine-5'-triphosphate (7-deaza-2'-dGTP) (Boehringer Mannheim, Indianapolis, IN) was included in several sequencing reactions at concentrations recommended by the manufacturer to resolve compressions. The DNA sequence was analyzed with the software provided by Genetics Computer Group (University of Wisconsin) or a VAX/VMS machine.

RNA Hybridization Analysis

Poly(A)⁺-RNA was isolated by affinity chromatography using oligo (dT)-cellulose (Theologis *et al.*, 1985). The RNAs were glyoxylated at 50°C, electrophoretically separated, blotted to GeneScreen membrane (DuPont, Wilmington, DE), and hybridized according to the manufacturer's instructions.

Determination of Transcription Initiation Site

The transcription initiation site of the OS-ACS1 gene was defined by primer extension with reverse transcriptase and by sequencing the primer extended product (Boorstein and Craig, 1989). Primer 5'- 32 P-racs1 (0.43 pmol) was hybridized with 2 μ g poly(A)⁺-RNA from uninduced or induced etiolated rice shoots at 55°C for 1 hr. The primer-RNA hybrids were incubated with 25 units of reverse transcriptase in 50 μ l of 1 mM deoxy nucleoside triphosphates (dNTPs), 10 mM dithiothreitol (DTT), 10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8.4), 60 μ g/ml Actinomycin D, 6 mM MgCl₂, and 20 units Ribonuclease inhibitor (Promega, Madison, WI) at 42°C for 1 hr. For sequencing the primer extended product, 40 μ g of poly(A)⁺-RNA from induced etiolated rice shoots were annealed to 8.6 pmol of 5'- 32 P-All primer as described above. The primer-RNA hybrids were extended with reverse transcriptase in the presence of dNTP/dideoxy nucleoside triphosphates (Calzone *et al.*, 1987). The products were analyzed on a 6% denaturing polyacrylamide gel.

Other Methods

The expression of prACS1 cDNA in *E. coli* was essentially as described (Rottmann *et al.*, 1991). Partial amino acid sequences deduced from PCR products corresponding to the N-terminal region of the ACC synthase protein (amino acid residues 60 to 91, see Figure 1, I) were aligned to generate a phylogenetic tree by using Parsimony After Progressive Alignment (PAPA) programs (Doolittle and Feng, 1990)

provided by Dr. Russell F. Doolittle (University of California, San Diego) and compiled on a SUN/UNIX computer.

Nomenclature of ACC Synthase Genes

At present, the nomenclature of the ACC synthase genes is in a confused state. Recently, a commission headed by Dr. Hans Kende (Plant Research Laboratory, Michigan State University) agreed that the ACC synthase genes from various plant species should be shown with the three letter symbol ACS (ACC Synthase) preceded by the Latin initials of each species. The genes are to be italicized and numbered chronologically according to deposition of the sequence in GenBank, with the exception of the tomato ACC synthase genes whose numbering will remain as specified by Rottman *et al.* (1991). The polypeptides encoded by the genes will be shown by the same symbols but not italicized. The *Arabidopsis thaliana* genes are to be shown by the same symbol but without the Latin initials prefix. Table 1 compares the old and new designations of the various genes as communicated in this paper.

RESULTS

Isolation of Genomic Sequences

Degenerate oligonucleotides (TZ-1F and TZ-2R) were synthesized based on the amino acid sequences MGLAENQ and FQDYHG, which are two highly conserved regions at the N-terminus of various ACC synthase isoenzymes (Figure 1, I). Using these degenerate primers with rice genomic DNA in the PCR, five DNA fragments migrate in two distinct regions of an agarose gel (P1 and P2). Region P1 has three, and P2 contains two fragments (Figure 1, IIIA). The P1 fragments were subcloned and 11 clones were sequenced. Three unique fragments, pfOS-1, pfOS-2, and pfOS-3 were identified, and each of them had significant sequence identity to each other (50–44%) and to other ACC synthases in that region (Figure 1, I). By screening 10^5 recombinant phages of a rice genomic library, we isolated 10 positive λ clones using the pfOS-1, pfOS-2, and pfOS-3 as probes. The clones fell into three groups of overlapping sequences. The partial restriction maps of three clones λ 10B1, λ 9A1b, and λ 13C1 representing the three groups are shown in Figure 1, II.

Southern analysis was carried out to demonstrate whether the three cloned PCR fragments represent all the ACC synthase sequences represented by the fragments that migrate in the P1 region. All the fragments from the P1 region were isolated, labeled, and used as probe for Southern analysis with genomic DNA. The results show that these fragments hybridized to three genomic fragments (Figure 1, IIIB), suggesting the presence of three genes. Similar analysis shows that the genomic PCR fragments pfOS-1, pfOS-2, and pfOS-3 also hybridize to the same three genomic fragments detected by the P1 region of the gel (Figure 1, compare IIIB with IIIC–E). Southern analysis with phage λ DNA of the clones shown in Figure 1, II, using the genomic PCR fragments as probes shows that the λ clones contain sequences that correspond to the same three genomic fragments to which the probes hybridize (Figure

Table 1. Nomenclature of the ACC synthase genes

Name of gene		Plant	Botanical name	Latin initials	References
Old	New				
CP-ACC1A, 1B	CP-ACS1A, 1B	Zucchini	<i>Cucurbita pepo</i>	CP	Sato and Theologis, 1989; Huang <i>et al.</i> , 1991
LE-ACC1A, 1B	LE-ACS1A, 1B	Tomato	<i>Lycopersicon esculentum</i>	LE	Rottmann <i>et al.</i> , 1991
LE-ACC2, pcVV4A, ACCSYN1, pBTAS1	LE-ACS2	Tomato	<i>Lycopersicon esculentum</i>	LE	Rottmann <i>et al.</i> , 1991; Van Der Straeten <i>et al.</i> , 1990; Olson <i>et al.</i> , 1991; Yip <i>et al.</i> , 1992
LE-ACC3, pBTAS2	LE-ACS3	Tomato	<i>Lycopersicon esculentum</i>	LE	Rottmann <i>et al.</i> , 1991; Yip <i>et al.</i> , 1992;
LE-ACC4, pcVV4B, ACCSYN2, pBTAS4	LE-ACS4	Tomato	<i>Lycopersicon esculentum</i>	LE	Rottmann <i>et al.</i> , 1991; Van Der Straeten <i>et al.</i> , 1990; Olson <i>et al.</i> , 1991; Yip <i>et al.</i> , 1992
pBTAS3	LE-ACS5	Tomato	<i>Lycopersicon esculentum</i>	LE	Yip <i>et al.</i> , 1992
pCMW33	CM-ACS1	Winter squash	<i>Cucurbita maxima</i>	CM	Nakajima <i>et al.</i> , 1990
pCMA101	CM-ACS2	Winter squash	<i>Cucurbita maxima</i>	CM	Nakagawa <i>et al.</i> , 1991
pAAS2	MS-ACS1	Apple	<i>Malus sylvestris</i>	MS	Dong <i>et al.</i> , 1991
CARACC3	DC-ACS1	Carnation	<i>Dianthus caryophyllus</i>	DC	Park <i>et al.</i> , 1992
TACC-13	NT-ACS1	Tobacco	<i>Nicotiana tabacum</i>	NT	Bailey <i>et al.</i> , 1992
ACC1, AT-ACC1	ACS1	Arabidopsis	<i>Arabidopsis thaliana</i>	AT	Liang <i>et al.</i> , 1992; Van Der Straeten <i>et al.</i> , 1992
ACC2, AT-ACC2	ACS2	Arabidopsis	<i>Arabidopsis thaliana</i>	AT	Liang <i>et al.</i> , 1992; Van Der Straeten <i>et al.</i> , 1992
ACC3, 4, 5	ACS3, 4, 5	Arabidopsis	<i>Arabidopsis thaliana</i>	AT	Liang <i>et al.</i> , 1992
OS-ACC1, 2, 3, 4, 5	OS-ACS1, 2, 3, 4, 5	Rice	<i>Oryza sativa</i>	OS	This work

1, compare IIIF–H with IIIB–E). The genomic sequences represented by these λ clones were designated as OS-ACS1, OS-ACS2, and OS-ACS3 (Figure 1, II). The chromosomal locations of OS-ACS1 and OS-ACS3 were determined by restriction fragment length polymorphism (RFLP) analysis in Dr. Steven Tanksley's laboratory (Cornell University) and is shown in Figure 1, II. The location of OS-ACS2 was not determined because its locus was not polymorphic.

The P2 fragments (Figure 1, IIIA) were also subcloned, and 10 clones were sequenced. Two unique fragments, pfOS-4 and pfOS-5, were identified, and each of them had significant sequence identity to each other (71%) and to three other rice ACC synthases (66–35%) in that region (Figure 1, I). They represent sequences from two additional divergent ACC synthases, OS-ACS4 and OS-ACS5. No further experiments have been carried out with these genomic PCR fragments.

Differential Expression Characteristics of OS-ACS Genes

We determined the expression of OS-ACS genes in etiolated rice seedlings in response to anaerobiosis and various other known inducers of ACC synthase gene expression, such as wounding, IAA, BzlAde, LiCl, CHX. The results are shown in Figure 2, I–III (A–C). For comparison, the expression of the rice Adhl gene (Xie and Wu, 1989) was also investigated (Figure 2, I–IIID). The plant hormone IAA induces only the OS-ACS1 gene (Figure 2, I, compare lanes 1 with 3 in A–C). LiCl a known potentiator of IAA-mediated ACC synthase mRNA induction (Boller, 1984; Sato and Theologis, 1989; Huang *et al.*, 1991) enhances the IAA-induced

expression of OS-ACS1 (Figure 2, IA, compare lane 3 with 5 in A, B, and C); LiCl alone has no effect (Figure 2, II compare lane 2 with 1 in A–C). Surprisingly, BzlAde, a known augmentor of IAA-induced ACC synthase activity (Yu *et al.*, 1979; Yoshii and Imaseki, 1981) and mRNA induction (Huang *et al.*, 1991) inhibits the induction of OS-ACS1 mRNA by IAA (Figure 2, IA, compare lanes 5 with 7).

Cycloheximide induces all three OS-ACS genes (Figure 2, II, compare lane 4 with 2 in A–C) and, more importantly, this protein synthesis inhibitor does not prevent the induction of OS-ACS1 by IAA/BzlAde/LiCl, indicating that the induction is a primary response (Ringold, 1979; Theologis *et al.*, 1985). Surprisingly, the induction of OS-ACS3 by CHX is enhanced by IAA/BzlAde/LiCl (Figure 2, IIC, compare lane 5 with 4). This result suggests that the OS-ACS3 gene is inducible by IAA/BzlAde/LiCl, but its inducibility is not detected, possibly because its mRNA is highly unstable. In the presence of CHX, however, the mRNA might be stabilized and become detectable. It remains to be determined whether IAA is the sole inducer of OS-ACS3 as it is for OS-ACS1 or whether it acts synergistically with BzlAde or Li or both to induce the OS-ACS3 gene. We attribute the OS-ACS1 and OS-ACS3 mRNA accumulation by IAA/BzlAde/LiCl to transcriptional activation; however, a posttranscriptional event may also be involved.

Anaerobiosis induces OS-ACS1 and OS-ACS3 gene expression (Figure 2, IIA and C, compare lane 7 with 6) and represses the expression of OS-ACS2 (Figure 2, IIC, compare lane 7 with 6). CHX superinduces the N₂-induced OS-ACS1 and OS-ACS3 mRNAs (Figure 2, III, compare lane 4 with 3 in A and C) suggesting that their induction is a primary response. The Adhl gene is in-

Figure 1. Isolation of genomic sequences. (I) Comparison of a highly conserved region (amino acid residues 53 to 97) of ACC synthase polypeptides among various plants. (a) Zucchini (Sato and Theologis, 1989; Huang *et al.*, 1991), (b) winter squash (Nakajima *et al.*, 1990; Nakagawa *et al.*, 1991), (c) tomato (Van Der Straeten *et al.*, 1990; Olson *et al.*, 1991; Rottman *et al.*, 1991; Yip *et al.*, 1992); (d) apple (Dong *et al.*, 1991), (e) carnation (Park *et al.*, 1992), (f) tobacco (Bailey *et al.*, 1992), (g) mung bean (Botella *et al.*, 1992, 1993), (h) *Arabidopsis* (Liang *et al.*, 1992; Van Der Straeten *et al.*, 1992), and (i) rice (this work). Completely conserved residues are shaded. Regions where the degenerate primers, TZ-1F and TZ-2R, have been designed are boxed. The vertical arrow indicates the position of an intron. The filled circle indicates one of the 11 invariant amino acids conserved between ACC synthase and aminotransferases (Rottmann *et al.*, 1991). Numbering of the residues is based on the zucchini pACS1 polypeptide (Sato *et al.*, 1991). Sequences derived from the DNA sequence of PCR fragments obtained with the TZ-1F and TZ-2R primers and rice genomic DNA or by sequencing the λ clones shown in panel II. (II) Restriction maps of OS-ACS genes. Solid bars indicate the fragments that contain the region corresponding to TZ-1F/TZ-2R PCR product. The direction of transcription is shown by an arrow; restriction sites are E, *EcoRI*; H, *HindIII*; S, *Sal I*; B, *BamHI*. (III) Southern analysis. (A) Ethidium bromide stained gel of amplification products generated by PCR with rice genomic DNA and TZ-1F/TZ-2R primers. (B–D) Southern analysis of genomic DNA (5 μ g/lane) digested with *EcoRI*/*Sal I*. Probes are 32 P-labeled TZ-1F/TZ-2R fragments generated from genomic DNA: P1, (B); pOS-1, (C); pOS-2, (D); pOS-3, (E). (F–H) Southern analysis of λ phage DNAs λ 10B1, (F); λ 9A1b, (G); and λ 13C1, (H). Probes are also 32 P-labeled PCR fragments.

duced only by N_2 , and its induction is also insensitive to CHX (Figure 2, IIID, compare lanes 3 with 1 and 4 with 3). Hybridization analysis with the rice 17S rRNA probe shows that the differential expression of OS-ACS genes cannot be attributed to uneven loading of the mRNAs in the various lanes (Figure 2, I–IIIE).

Tissue Specific Expression of OS-ACS Genes Under Anaerobic Conditions

To determine whether the OS-ACS1 and OS-ACS3 genes are differentially expressed in a tissue specific manner under anaerobic conditions, RNA hybridization analysis was carried out with mRNA isolated from shoots and roots of air- or N_2 -treated, eight-day-old, etiolated seedlings. The results are shown in Figure 3. The OS-ACS1 is induced only in the shoots after 12 h of anaerobic treatment (Figure 3A, compare lane 2 with 1), and its induction is nil in roots (Figure 3A, compare lane 4 with 3). In contrast, the OS-ACS3 is induced only in the roots under the same conditions (Figure 3C, compare lanes 1–4 with those in Figure 3A). The *Adh1* gene is induced in both shoots and roots after 12 h of N_2 treatment (Figure 3D, compare lane 2 with 1 and lane 4 with 3, respectively). The OS-ACS2 gene is primarily expressed in the roots of etiolated seedlings (Figure 3B,

Figure 2. Expression of ACC synthase genes in etiolated rice seedlings after treatment with IAA, BzlAde, LiCl, cycloheximide (CHX), and anaerobiosis (N_2). All panels are autoradiographs of RNA filters hybridized with the following 32 P-labeled probes. (A) *prACS1*, (B) *pfOS-2*, (C) *pfOS-3*, (D) *pRCA1* [rice *Adh1* cDNA (Xie and Wu, 1989)], (E) 17S rRNA (Takaiwa *et al.*, 1984). (I) Etiolated detached rice shoots treated for 18 hr as follows. Lanes: 1, uninduced; 2, LiCl; 3, IAA; 4, BzlAde; 5, LiCl + IAA; 6, BzlAde + LiCl; 7, IAA + BzlAde; 8, IAA+BzlAde+LiCl. (II) Etiolated detached rice shoots treated for 18 hr as follows. Lanes: 1, intact shoot; 2, uninduced; 3, IAA+BzlAde+LiCl; 4, CHX; 5, IAA+BzlAde+LiCl+CHX; 6, Air-12 hr; 7, N_2 -12 hr. (III) Intact etiolated seedlings treated for 12 hr as follows. Lanes: 1, Air; 2, Air+CHX; 3, N_2 ; 4, N_2 +CHX. All hybridizations were carried out with 2×10^6 cpm/ml hybridization mixture. All lanes in I and II contain 2 μ g polyA⁺-RNA/lane, except lanes 6 and 7, in II that contain 4 μ g/lane. Exposure times of autoradiographs in all panels are (A) 18 hr, (B) 18 hr, (C) 2 weeks, (D) 0.5 hr, and (E) 0.5 hr. All autoradiographs in I–III were obtained using the same RNA filters that were successively washed and reprobed. The 17S rRNA probe is a 400-bp *EcoRI*/*HindIII* fragment from the 5' end (158–557 bp) of the rice 17S rRNA gene (Takaiwa *et al.*, 1984). The fragment was synthesized by PCR.

Figure 3. Tissue specific expression of ACC synthase genes in etiolated rice seedlings after anaerobiosis (N_2). All panels are autoradiograms of RNA filters hybridized with the same ^{32}P -labeled probes mentioned in the legend of Fig. 2. Treatment: 8-d-old etiolated seedlings were treated for 12 h in air or N_2 and their roots and shoots (coleoptiles containing the primary leaves) were separated and used for polyA⁺-RNA isolation. The lanes are 1, air-shoots; 2, N_2 -shoots; 3, air-roots; 4, N_2 -roots. The amount of polyA⁺-RNA loaded in each lane is (A) and (B) 10 μ g, (C) 20 μ g, (D) 2 μ g, and (E) 2 μ g. All hybridizations were carried out with 2×10^6 cpm/ml hybridization mixture. Exposure time of autoradiograms are (A) 42 h, (B) 42 h, (C) 8 d, (D) 3 h, and (E) 3 h. All autoradiograms were obtained with different RNA filters.

compare lane 3 with 1), and its expression is suppressed by N_2 treatment (Figure 3B, compare lanes 1 and 3 with 2 and 4).

Structure of OS-ACS1

Among the three ACC synthase genes, the expression of only OS-ACS1 is greatly potentiated by anaerobiosis (Figure 2, IIA and IIIA). Because we are interested in understanding how hypoxia induces ACC synthase activity at the molecular level, the OS-ACS1 gene was chosen for further structural characterization. The sequence of 2778 nucleotide (nt) of the gene has been determined and is shown in Figure 4. The gene consists of four exons and three introns. The sequence also includes part of the 5'- and 3'-flanking regions. The nucleotide sequence of the prACS1 cDNA (1482 bp) is identical to those of the OS-ACS1 exons except for single nucleotide changes at positions 386, 452, and 819. At position 386, there is an A instead of a C, encoding Asn 129 (instead of Thr 129). At position 452, there is a T instead of a C, encoding Phe 151 (instead of Leu 151). At position 819, there is a G instead of an A, encoding Gly 273 (instead of Asp 273). Because the cDNA was obtained by PCR these changes probably reflect errors during PCR amplification.

Determination of the Transcription Initiation Site

The transcription start site was determined by primer extension using reverse transcriptase (Figure 5). Furthermore, primer extension performed in the presence of dideoxynucleotides revealed an mRNA sequence

identical to the genomic sequence found upstream from the prACS1 cDNA (compare Figure 5, B and C).

Expression of OS-ACS1 Polypeptide

The authenticity of the OS-ACS1 polypeptide was confirmed by expression experiments in *E. coli*. The prACS1 cDNA was synthesized by PCR and was fused to the N-terminus of the β -galactosidase gene in pUC18 in sense (prACS50) and pUC19 in antisense (prACS51) orientation (see MATERIALS AND METHODS). *E. coli* transformants bearing the prACS50 plasmid produce 7 nmol ACC/ 10^8 cells after 1 hr of incubation in the presence of 1 mM isopropylthiogalactose (IPTG) (Rottmann *et al.*, 1991). *E. coli* containing only pUC18 or prACS51 does not accumulate ACC.

DISCUSSION

Ethylene and Anaerobiosis

We used a PCR-based approach for cloning the first genomic sequences encoding ACC synthase from rice, a monocotyledonous plant. The results indicate that in rice ACC synthase is encoded by a gene family that consists of at least five divergent members. Similar results were found for the ACC synthase genes from various dicotyledonous plants such as tomato (Rottmann *et al.*, 1991) and *Arabidopsis* (Liang *et al.*, 1992).

Our data indicate that a number of distinct regulatory networks govern the expression of different rice ACC synthase genes under various hormonal and environmental signals. This differential pattern of accumulation reflects the ability of the ethylene biosynthetic pathway to respond to diverse array of developmental and environmental signals associated with the production of ethylene, which affects many aspects of plant growth, development, and disease resistance (Yang and Hoffman, 1984; Abeles *et al.*, 1992).

The demonstration that ACC synthase genes are expressed under anaerobic conditions supports the large body of physiological evidence linking ethylene with growth of rice under low O_2 tension. Past work with rice has shown that hypoxia increases the concentration of ethylene and promotes growth of several organs including coleoptile, mesocotyl, root, and stem (Jackson *et al.*, 1987) and enhances aerenchyma formation in adventitious roots (Justin and Armstrong, 1991). The tissue specific expression of OS-ACS1 and OS-ACS3 under anaerobiosis (Figure 3) suggests that different ACS isoenzymes are responsible for mediating the ethylene regulated growth responses in various organs. The isolation of these genes will allow the construction of rice mutants by reverse genetics for defining the role of ethylene in the growth process. It will also allow the determination of the cell types and tissues involved in ethylene mediated growth. Anatomical analysis of growth in the internode of deepwater rice has shown

Figure 4. DNA sequence of the *OS-ACS1* gene including introns and 5' and 3' flanking regions. The nucleotide at position +1 corresponds to the transcription initiation site (Figure 5). The nucleotides upstream from the +1 position are negatively numbered. The sequence of the mRNA transcribed by the gene is shown in capital letters. The cDNA sequence is shaded; the remainder of the sequence is in small letters. The derived amino acid sequence is presented in the one-letter code below the DNA sequence and is numbered separately. The 11 boxed residues are the invariant amino acids conserved between ACC synthase and various aminotransferases (Rottmann *et al.*, 1991). The underlined dodecapeptide beginning at Ser 283 is part of the active center of ACC synthase (Yip *et al.*, 1990). The underlined bases beginning at position +64 show the *rac1* primer (20mer) used for determining the start of transcription (Figure 5). The TATA box and a putative polyadenylation signal sequence are boxed.

It is of great interest that the plant hormone IAA induces the anaerobically regulated ACC synthase genes, indicating a possible link between anaerobiosis and auxin. A similar link has been established for the *Adhl* gene. The ADH activity in maize and rice is induced by N₂ and auxin (Freeling, 1973; Xie and Wu, 1989). The possibility exists that certain auxin mediated growth responses are mediated by ethylene.

The monovalent cation Li^+ is one of the strongest effectors of ACC synthase gene expression (Sato and Theologis, 1989; Huang *et al.*, 1991; Liang *et al.*, 1992). It also exerts a profound effect on both human behavior and early embryonic development. Recent experimental evidence indicates that LiCl interferes with the formation of inositol phosphates (Berridge *et al.*, 1989). Because plant tissues are never exposed to 50 mM LiCl (a concentration used in this and other studies), it is likely that LiCl simply unmask preexisting biochemical capacity present in the plant cells that is normally utilized by other natural transducers such as hormones and environmental signals.

Figure 5. Determination of the transcription initiation site of *OS-ACS1*. (A) Primer extension analysis. $5'$ - 32 P-racs1 primer (Figure 4) was hybridized with 2 μ g polyA $^{+}$ -RNA from uninduced-18 hr (lane 1), or induced-18 hr etiolated rice shoot tissue (lane 2), and extended with reverse transcriptase. (B) Dideoxy sequencing reactions with ssDNA of the 1.5-kb *EcoRI/Sal I* fragment from *OS-ACS1* (Figure 1, II) using racs1 primer. Lanes 1–4 are reactions A, C, G, and T, respectively. (C) Nucleotide sequencing of the 5' end of the mRNA. $5'$ - 32 P-racs1 primer was hybridized with 40 μ g of poly A $^{+}$ -RNA from induced-18 hr etiolated rice shoot tissue and extended with reverse transcriptase in the presence of dNTP/ddNTPs (Boorstein and Craig, 1989). Lanes 1–4 are like those in B. The bold residue represents the major start of transcription.

The induction of the ACC synthase genes by CHX appears to be widespread (Huang *et al.*, 1991; Liang *et al.*, 1992). Protein synthesis inhibition has been shown to enhance the level of various hormonally regulated mammalian genes whose products are regulatory proteins such as oncogenes (Atwater *et al.*, 1990), as well as of some auxin regulated genes in pea and soybean (Theologis *et al.*, 1985; Franco *et al.*, 1990). It appears that cycloheximide inducibility is linked to genes whose products are unstable and have regulatory function. Throughout the years two mechanisms have been proposed to explain CHX inducibility. First, the gene may

be under the control of a short-lived repressor protein whose synthesis is prevented by addition of CHX (Sen and Baltimore, 1986). Second, protein synthesis inhibition may also prevent mRNA degradation by preventing the synthesis of a labile nuclease. CHX has a dual effect on the induction of the early auxin regulated genes IAA4/5 and IAA6 in pea (Theologis *et al.*, 1985), activation of transcription and mRNA stabilization (Koshiba, Ballas, and Theologis, unpublished observations). The effect of CHX on *OS-ACS1* and *OS-ACS2* can be explained by the dual mechanism of CHX action. However, the induction of *OS-ACS3* by CHX is probably due to mRNA stabilization. The differential accumulation of *OS-ACS* mRNAs in the presence of CHX may reflect differential rate of transcription as well as differential mRNA stability among the three genes.

Structural Features of *OS-ACS1*

The coding region of the gene consists of 1464 bp which is interrupted by three introns (Figure 4). The intron/exon junctions, which are typical of donor and acceptor splice sites, have been established by reference to the sequence of prACS1 cDNA (Breathnach and Chambon, 1981). The number and size of exons and the location of introns is similar to other ACC synthase genes isolated from zucchini, tomato, and *Arabidopsis* (Huang *et al.*, 1991; Rottmann *et al.*, 1991; Liang *et al.*, 1992), except that the zucchini ACC synthase has an additional intron within the last exon (Huang *et al.*, 1991). We have defined the transcription start site of *OS-ACS1* gene by primer extension and by sequencing the primer extended product (Figure 5, A and C). The mRNA sequence determined by RNA sequencing is identical to the genomic sequence found in the 5' untranslated region of *OS-ACS1* (Figure 5, B and C) indicating that there is no additional intervening sequences in that region. There is a sequence beginning at position –30 (Figure 4) that qualifies as a TATA box (Breathnach and Chambon, 1981). The 3' end of the gene was determined by sequencing an IPCR product containing the 5' and 3' ends of the mRNA. A potential polyadenylation signal (AATAAA) is located 31 nucleotides upstream from the polyadenylation site (Figure 4). The mRNA transcribed by the *OS-ACS1* gene is predicted to be 1976 nt long (5', 167 nt; coding region, 1464 nt; 3', 345 nt) without poly A tail, close to the size of 2000 nt detected by northern analysis (unpublished observations).

The *OS-ACS1* polypeptide is comprised of 487 amino acids (M_r 53,112 Da; pI 8.2) close to the size of other ACC synthase isoenzymes from various plant species (Rottman *et al.*, 1991). It is most similar in primary sequence (68% identity) as well as in size and isoelectric point to those of *LE-ACS3*, the most divergent member of the tomato ACC synthase multigene family (Rottmann *et al.*, 1991). The rice polypeptide also contains all the conserved regions found in other ACC synthase

Figure 6. Phylogenetic tree of ACC synthase. The dendrogram was constructed from the alignment shown in Figure 1, I (without the TZ-1F and TZ-2R conserved regions) using the PAPA method (Doolittle and Feng, 1990). The numbers in parentheses are percent identities of the sequences at the tips beyond. The genes underlined and shown in bold are auxin-inducible in vegetative tissues (see DISCUSSION). The rice genes are shaded.

isoenzymes from various dicotyledonous plants (Rottmann *et al.*, 1991; Theologis, 1992) as well as the 11 invariant amino acids conserved between ACC synthases and aminotransferases (Figure 4). The dodecapeptide sequence characteristic of the pyridoxal phosphate binding site is also present (Figure 4).

Evolution of ACC Synthase

Phylogenetic analysis of amino acid sequences shown in Figure 1, I using the PAPA methods (Doolittle and Feng, 1990) yields an unrooted phylogenetic tree shown in Figure 6. The tree shows that some ACC synthase genes are more similar to genes of other species than to other ACS genes in the same species. One major lineage contains OS-ACS1 along with five other genes from winter squash (Nakagawa *et al.*, 1991), tomato (Rottmann *et al.*, 1991), *Arabidopsis* (Liang *et al.*, 1992), and apple (Dong *et al.*, 1991). Similar analysis with the PHYLIP program using the complete coding sequence of 20 ACC synthase genes reveals a nearly identical genealogy (unpublished observation). Furthermore, the tree indicates early trifurcation of the ACS genes into

three main branches with subsequent divergence within each class accompanying the evolution of monocots and dicots. Based on the results of phylogenetic analysis, we propose that the polymorphism of ACC synthase arose before the time of divergence of monocotyledonous and dicotyledonous plants and is likely to be present in all higher plants. An early evolutionary origin has also been found for the phytochrome gene family, the most ancient regulatory photoreceptor family in plants (Dehesh *et al.*, 1991). The ACC to ethylene pathway has been detected in cycads and in certain ferns (Liang *et al.*, 1992). Thus, the evolution of ACC synthase could have begun over 350 million years ago, much more than the estimated 125–150 million years since monocots and dicots diverged (Martin *et al.*, 1989). Moreover, the current data indicate a striking correlation for some ACC synthase genes between their phylogenetic relationship and their pattern of expression. For example, the genes that are induced by auxin in vegetative tissues, including OS-ACS1 (this paper), CM-ACS2 from winter squash (Nakagawa *et al.*, 1991), ACS4 and ACS5 from *Arabidopsis* (Liang *et al.*, 1992), and LE-ACS3 from tomato (Lincoln and Theologis, unpublished observations) all fall into one major lineage (Figure 6). The possibility exists that the generation of separate regulatory networks controlling the expression of ACC synthase subfamilies is also an early evolutionary event preceding the divergence of monocots and dicots.

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