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Apple Ripening-Related cDNA Clone pAP4 Confers Ethylene-Forming Ability in Transformed *Saccharomyces cerevisiae*[1]

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The apple ripening-related cDNA insert of clone pAP4 (G.S. Ross, M.L. Knighton, M. Lay-Yee [1992] Plant Mol Biol 19: 231–238) has previously been shown to have considerable nucleic acid and predicted amino acid sequence similarity to the insert of a tomato ripening-related cDNA clone (pTOM13) that is known to encode the enzyme 1-aminocyclopropane-1-carboxylate (ACC) oxidase (A.J. Hamilton, G.W. Lycett, D. Grierson [1990] Nature 346: 284–287; A.J. Hamilton, M. Bouzayen, D. Grierson [1991] Proc Natl Acad Sci USA 88: 7434–7437). The cDNA insert from the clone pAP4 was fused between the galactose-inducible promoter and the terminator of the yeast expression vector pYES2. Transformation of *Saccharomyces cerevisiae* strain F808– with this DNA construct and incubation of the yeast in the presence of D[+]-galactose allowed these cells to convert ACC to ethylene. The transformed yeast converted 1-amino-2-ethylcyclopropane-1-carboxylate isomers to 1-butene with the same 1*R*,2*S*-stereoselectivity as achieved by the native ACC oxidase from apples. Both ascorbate and Fe^{2+} ions stimulated the rate of the production of ethylene from ACC by the transformed yeast, whereas Cu^{2+} and Co^{2+} were strongly inhibitory; these are features of ACC oxidase. Northern analysis of the total RNA from nontransformed and transformed yeast showed that the ability to convert the ACC to ethylene was correlated with the synthesis and accumulation of a novel 1.2-kb mRNA that hybridized to the cDNA clone pAP4. We conclude that the cDNA sequence of the clone pAP4 encodes ACC oxidase.

Ethylene is significantly important in many stages of plant development and growth. It is becoming increasingly apparent that the biosynthesis of ethylene is developmentally regulated and temporally activated in response to numerous biotic and abiotic factors. An increase in the level of synthesis of this simple olefin occurs during leaf and flower petal senescence and the ripening of climacteric fruits and, in many tissues, following periods of temperature, wounding, or anaerobic stress (Yang and Hoffman, 1984). Ethylene is synthesized from Met via the intermediates *S*-adenosyl-L-methionine and ACC, which is converted to ethylene by the terminal biosynthetic enzyme ACC oxidase (Adams and Yang, 1979; Yang and Hoffman, 1984). In ripening climacteric fruit, the biosynthesis of ethylene is autocatalytic in that it promotes the accumulation of ACC synthase and ACC (Yang and Hoffman, 1984).

Fruit ripening is accompanied by altered levels of many different mRNAs and their encoded proteins (Christoffersen et al., 1984; Grierson et al., 1985). The application of exogenous ethylene to mature climacteric fruit, not yet stimulated to produce ethylene, is known to stimulate the ripening process and cause the accumulation of many of these mRNAs and the proteins they encode (Grierson and Tucker, 1983; Grierson et al., 1985; Maunders et al., 1987), including those of the ethylene biosynthetic pathway.

ACC synthase has been purified and characterized from a number of different tissues (Bleecker et al., 1986; Nakajima et al., 1988; Van Der Straeten et al., 1990; Sato et al., 1991; Yip et al., 1991), and cDNA clones have been identified for this protein (Van Der Straeten et al., 1990; Olson et al., 1991). However, it is only recently that authentic ACC oxidase has been obtained from plant material as an active enzyme (Smith et al., 1991; Kuai and Dilley, 1992) and purified to homogeneity in an active form (Dong et al., 1992a; Dupille et al., 1992). The cDNA clone for tomato ACC oxidase (pTOM13) has recently been identified from a ripening-related library (Slater et al., 1985), by the application of antisense technology (Hamilton et al., 1990), and by expression in yeast (Hamilton et al., 1991). A cDNA clone for tomato ACC oxidase has also been identified by expression in *Xenopus laevis* oocytes (Spanu et al., 1991). cDNA clones showing a high degree of nucleotide sequence homology to pTOM13 have also been isolated for apples (pAP4 [Ross et al., 1992] and pAe12 [Dong et al., 1992b]), avocados (pAVOe3 [McGarvey et al., 1990]), and carnation flowers (pSR120 [Wang and Woodson, 1991]). The importance of ethylene in climacteric fruit ripening has been clearly demonstrated by the retardation of the normal ripening process in the fruit of transgenic plants that express antisense RNA for ACC synthase (Oeller et al., 1991) or ACC oxidase (Hamilton et al., 1990) and show grossly reduced levels of ethylene synthesis.

Recent work in our laboratory (Dilley et al., 1992) and others (Dong et al., 1992a) has shown that the activity of purified apple fruit ACC oxidase has an absolute requirement for CO_2 in the gaseous state, utilizes ascorbic acid as a co-substrate with ACC, and requires Fe^{2+}. More important, the activity of the enzyme is dependent on the presence of CO_2

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Abbreviation: AEC, 1-amino-2-ethylcyclopropane-1-carboxylic acid.

in a concentration-dependent manner. The mechanism by which this occurs is not known, but it may be achieved via the carbamylation of specific Lys residues within the protein as is known to occur for Rubisco (Lorimer and Miziorko, 1980).

A prerequisite for determining the CO_2 activation mechanism for ACC oxidase is the construction of an artificial system for the in vivo expression of the normal ACC oxidase cDNA and its site-directionally mutated derivatives. Here we present further evidence, by expression in the yeast strain F808−, a model system for studying the effects of the site-directed mutagenesis of this enzyme, that the cDNA sequence of the ripening-related apple clone pAP4 (Ross et al., 1992) does indeed encode ACC oxidase. This sets the stage for a series of experiments that may determine which functional domains of ACC oxidase are involved in the observed CO_2 activation.

MATERIALS AND METHODS

Cloning of pAP4 cDNA Insert into pYES2

All of the DNA manipulations and cell transformations were carried out using standard techniques (Maniatis et al., 1989). The yeast plasmid expression vector pYES2 (Invitrogen) and the plasmid pAP4 (Ross et al., 1992), an 1182-bp apple ripening-related cDNA cloned 5′ to 3′ into the *Not*I and *Sal*I sites of the vector pSPORT (BRL), were subjected to sequential and complete digestion with the restriction endonucleases *Xba*I and *Kpn*I (Stratagene). The larger linearized fragment of pYES2 (5773 bp) and the 1221-bp cDNA-containing fragment of pAP4 were purified from 1% (w/v) agarose gels using the Prep-a-Gene system supplied by Bio-Rad and were ligated using T4 DNA ligase (Stratagene) so as to insert the cDNA sequence directionally between the Gal-inducible promoter and the terminator of the pYES2 plasmid. *Escherichia coli* DH5α (GIBCO-BRL), transformed with the ligation products, were selected on agar plates containing 50 μg mL^{-1} of ampicillin. An *E. coli* transformant containing a pYES2 plasmid with the pAP4 cDNA (termed pAPY4) was chosen for further experiments.

Growth and Transformation of *Saccharomyces cerevisiae*

The yeast (*S. cerevisiae*) strain F808− (Gal^+, Leu 2–3, Leu 2–112, His 4–519, Ade 1–100, Ura 3–52) was obtained from Dr. Hans Kende, Michigan State University, as individual colonies growing on 1.5% (w/v) Bacto-agar (Sigma) plates containing YPD (1% [w/v] yeast extract [Sigma], 1% [w/v] Bacto-peptone [Difco], 1% [w/v] dextrose) medium (Sherman, 1990).

The yeast cells from an individual colony were grown and transformed with pAPY4 DNA by electroporation (Becker and Guarente, 1990) using a single 5-ms pulse (E_o = 7.5 kV cm^{-1}, 25 μF, 200 Ω) and a Bio-Rad Gene Pulser apparatus. Selection of transformed yeast cells was achieved by plating on 1.5% (w/v) Bacto-agar containing 1 M sorbitol and yeast selection medium with Glc (YSM_{glu}) (0.65% [w/v] yeast nitrogen base without ammonium sulfate or amino acids [Difco], 2% [w/v] Glc, 0.5% [w/v] ammonium sulfate, 470 mg L^{-1} of dry amino acid mix, and 50 μg mL^{-1} of ampicillin) and incubating at 30°C for 48 h. Dry amino acid is 8.8% (w/w) adenine, 4.2% (w/w) Arg, 21.2% (w/w) Asp, 4.2% (w/w) His, 12.7% (w/w) Leu, 6.4% (w/w) Lys, 4.2% (w/w) Met, 10.6% (w/w) Phe, 12.8% (w/w) Thr, 8.5% (w/w) Trp, and 6.4% (w/w) Tyr.

Assay for Ethylene-Forming Ability of Transformed Yeast

Individual segregated colonies of yeast F808−, transformed with pAPY4, were picked from the YSM_{glu} plates and used to inoculate separate 100-mL aliquots of liquid YSM_{gal} medium (YSM medium containing 2% [w/v] Gal instead of Glc), and the cultures were incubated with shaking at 30°C until the A_{600} approximated 1.4 (approximately 1×10^8 cells mL^{-1}). Ten 980-μL replicate aliquots of each of the cultures of transformed yeast cells were placed in individual 5-mL vials, prewarmed to 30°C. To each vial was added 10 μL of 100 mM ACC, 6 μL of freshly prepared 0.5 M ascorbic acid, and 4 μL of freshly prepared 1 mM $FeSO_4$. The vials were immediately capped with a Subaseal vaccine cap and were incubated at 30°C for 1 h with shaking at 225 rpm. Following incubation, 1.0 mL of the head space was assayed for ethylene by GC using a Hach-Carle series 100 AGC. Nontransformed yeast grown in YPD medium supplemented with 2% (w/v) D[+]-Gal and transformed yeast grown in YSM_{glu} medium were similarly assayed for their ability to oxidize ACC.

Determination of Optimum Yeast Growth Phase for Ethylene-Forming Ability

Ten milliliters of the culture suspension of transformed yeast that showed the best ability to convert ACC to ethylene were used to inoculate 250 mL of prewarmed YSM_{gal} medium, and the inoculum was shaken at 30°C. At intervals of 90 min, the A_{600} of the culture was determined, and 10 replicate 980-μL aliquots of the growing yeast culture were assayed for ethylene-forming ability as before. Subsequent studies of ethylene production were performed with cultures reaching the A_{600} at which the maximum ethylene-forming ability was attained. A similar time-course analysis was performed with nontransformed yeast growing in YPD medium supplemented with 2% (w/v) D[+]-Gal.

Analysis of Stereospecificity and the Effects of Heavy Metal Ions, Ascorbate, and Fe^{2+} on the Ethylene-Forming Ability of the Transformed Yeast

The transformed yeast cells containing ACC oxidase were assayed for substrate stereospecificity by substituting a racemic mixture of either *trans*-(1*R*,2*S* and 1*S*,2*R*)- or *cis*-(1*R*,2*R* and 1*S*,2*S*)-AEC for the ACC in the standard ethylene assay and measuring the 1-butene produced after 2 to 3 h of incubation. Olefin identity was assessed on the basis of the cochromatography of known gas standards. The effect of Co^{2+} or Cu^{2+} on the ability of the yeast to make ethylene was determined by including them in the standard assay at a final concentration of 25 μM. Standard ethylene assays were also performed with and without ascorbate and/or Fe^{2+} to determine the contribution of these ions with regard to the ability of the yeast to synthesize this olefin.

Nucleic Acid Analysis

Total RNA was extracted from the nontransformed yeast when the A_{600} of a culture in YPD medium supplemented with 2% (w/v) D[+]-Gal was equal to 1.2 and from transformed yeast grown in YSM_{gal} at successively increasing A_{600} values. In each case, approximately 3.7×10^7 cells were collected by centrifugation (1000*g*, 4°C, 10 min), and the pellets were resuspended in 5 mL of 1.2 M sorbitol at 20°C. Spheroplast formation was achieved by adding 5000 units of lyticase (Sigma) in 50 mM potassium phosphate (pH 7.5) and incubating at 37°C for 20 min. Subsequently, 5 mL of extraction buffer (0.1 M Tris-HCl [pH 8.0], 1% [w/v] triisopropylnaphthalene sulfonic acid [Kodak], 6% [w/v] *p*-aminosalicylic acid, 5% [v/v] water-saturated phenol, and 1% [w/v] 8-hydroxyquinoline) were added, and the mixture was shaken gently for 1 min and partitioned twice against phenol: chloroform (1:1). The total nucleic acid was precipitated from the aqueous phase by the addition of 2.5 volumes of ethanol (−20°C, 30 min) and pelleted by centrifugation (12,000*g*, 30 min, −10°C). The total RNA was selectively purified from the total nucleic acid by repeated precipitation from 3 M sodium acetate (pH 5.6) and centrifugation at 4°C. The final RNA pellet was washed successively with 50 mM potassium acetate in 80% (v/v) ethanol, 80% (v/v) ethanol, and 95% (v/v) ethanol, dried under vacuum, dissolved in sterile distilled water at a final concentration of 5 $\mu g\ \mu L^{-1}$, and stored at −70°C.

Each RNA sample (20 μg) was subjected to denaturing electrophoresis in 1% (w/v) agarose gels and blotted onto 0.2-μm nylon transfer membranes (Micron Separations Inc.) (Maniatis et al., 1989). The bound RNA was hybridized at 65°C for 18 h with approximately 30 ng of randomly primed (Boehringer Mannheim) [^{32}P]dCTP-labeled (approximately 1.2×10^9 dpm μg^{-1}) pAP4 cDNA insert (Maniatis et al., 1989). After hybridization, the filters were washed successively in 300 mL of 0.1× SSPE (20 × SSPE is 3 M NaCl, 0.2 M NaH_2PO_4 [pH 7.4], 25 mM EDTA), 0.1% (w/v) SDS for 30 min at 65°C, and then in another 300 mL of the same solution at room temperature. The washed filters were subjected to autoradiography with X-OMAT (AR) x-ray film (Kodak).

RESULTS

Using the method of Becker and Guarente (1990), electroporation of *S. cerevisiae* strain F808− in 1 M sorbitol with purified pAPY4 DNA resulted in the production of 7.2×10^3 colony-forming units μg^{-1} of DNA used. Complementation of the Ura3 mutation in *S. cerevisiae* F808− by a functional gene on pAPY4 allowed selection of transformants on medium without uracil. Ten individually segregated transformed colonies (termed pAPY4a to pAPY4j) were cultured in YSM_{gal} medium with 50 $\mu g\ mL^{-1}$ of ampicillin until the A_{600} approximated 1.4, and their competency to oxidize ACC to ethylene was measured. In the presence of ascorbate and Fe^{2+}, all of the cultures of yeast transformed with pAPY4 and grown in YSM_{gal} medium, but not nontransformed yeast grown in YPD medium supplemented with 2% (w/v) D[+]-Gal, were able to convert ACC to ethylene. Of these, pAPY4b reproducibly showed the highest ethylene production in replicate assays, and this culture was used for all subsequent investigations. Transformed yeast grown in YSM_{glu} medium failed to produce ethylene in the standard assay (data not shown).

In the growth time-course analysis of yeast clone pAPY4b (Fig. 1), the ethylene-forming ability of the cells initially increased following inoculation of fresh YSM_{gal} medium and then declined as the culture entered the early exponential phase of growth. During the midexponential phase, the ability of the cells to oxidize ACC to ethylene increased and reached a maximum in the late exponential phase, after which it rapidly declined as the culture entered the stationary growth phase. Northern analysis of total RNA extracted from both the transformed and nontransformed yeast demonstrated that, when grown in the presence of Gal, only the transformed yeast cells synthesized a novel 1.2-kb mRNA that hybridized to the cDNA insert from the plasmid of the clone pAP4 (Fig. 2). This mRNA showed an increase in abundance during the growth of the transformed yeast cells, reaching a maximum level coincident with the growth stage at which the cells reattained their greatest ethylene-forming ability. Thereafter, the level of the mRNA declined. Both chromosomal and plasmid DNA were absent from all of the total RNA preparations, as shown by standard agarose gel electrophoretic analysis (data not shown).

The ethylene-forming ability of the yeast transformed with pAPY4 was tested for stereoselectivity of substrate by incu-

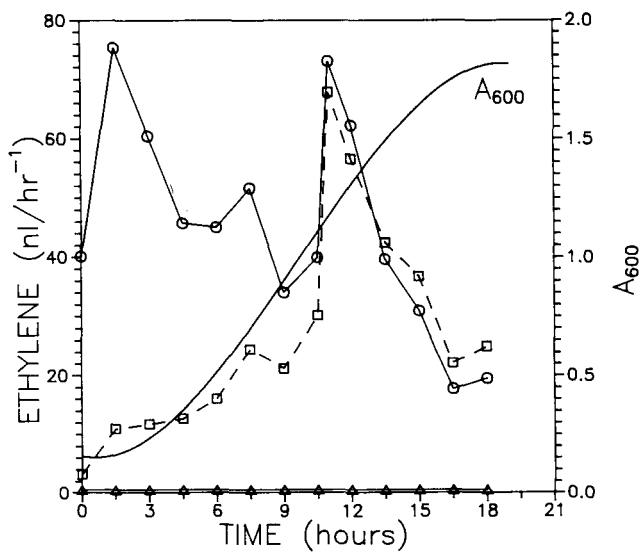

Figure 1. Growth and ethylene production capacity of nontransformed yeast and yeast transformed with pAPY4. Prewarmed YPD medium supplemented with 2% (w/v) D[+]-Gal or YSM_{gal} medium was inoculated with 10 mL of mid-log phase nontransformed or transformed yeast cells, respectively, and the cultures were shaken at 225 rpm and 27°C. At 90-min intervals, both the transformed (○) and nontransformed (△) cultures were assayed in the presence of ascorbate and Fe^{2+} for ethylene-forming ability as described in the "Materials and Methods." In each of these cases, the ethylene production is expressed per 10^8 cells. The total ethylene produced by the transformed yeast at each assay time (□) is also shown, as is the growth curve of the yeast as determined by their A_{600} spectra. Each measurement represents the mean of 10 replicates.

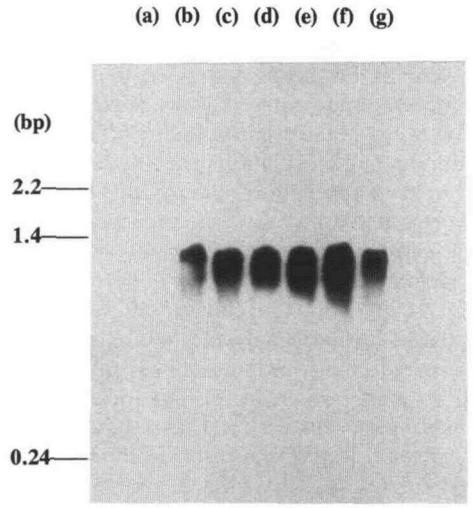

Figure 2. Northern analysis of the RNA from nontransformed yeast F808− and yeast transformed with pAPY4. Northern analysis of 20 μg of total RNA from nontransformed yeast F808- grown in YPD medium supplemented with 2% (w/v) D[+]-Gal at A_{600} equal to 1.2 (a) and from yeast F808− transformed with pAPY4 and grown in YSM_{gal} at A_{600} equal to 0.25 (b), 0.5 (c), 0.75 (d), 0.96 (e), 1.17 (f), and 1.5 (g). Total RNA was extracted, subjected to gel electrophoresis, transferred to nylon membrane filters, and hybridized to radiolabeled pAP4 cDNA insert as described in the "Materials and Methods." The filters were washed as described, and the results were viewed by autoradiography.

bating aliquots of the culture with either the *trans*- or *cis*-isomers of AEC. The transformed yeast stereoselectivity converted the *trans*-AEC isomer to 1-butene in preference to the *cis*-AEC isomer, which was converted to 1-butene at a much lower rate (Table I). Cu^{2+} and Co^{2+}, which are known to inhibit ACC oxidase, significantly reduced the conversion of ACC to ethylene by the transformed yeast cells (Table II). ACC oxidase requires ascorbate and Fe^{2+}. Deleting ascorbate from the assay mixture reduced the level of ethylene production by the transformed yeast by approximately 50% (Table III). Deleting Fe^{2+} from the assay produced a less marked reduction in the ability of the yeast to produce ethylene (Table III). Deleting both ascorbate and Fe^{2+} from the assay diminished ethylene production to about 38% of the control value.

Table I. *Stereoselectivity of yeast transformed with pAPY4 to produce 1-butene from isomers of AEC*

AEC Isomer	1-Butene Produced	
	2-h incubation	3-h incubation
	nL of 10^8 cells^{-1} h^{-1}	
trans-(1*R*,2*S* and 1*S*,2*R*)-AEC	126	299
cis-(1*R*,2*R* and 1*S*,2*S*)-AEC	4[a]	6[a]

[a] *F* test for comparison of means within incubation times is significant at $P \leq 0.01$. Transformed yeast cells were grown in YSM_{gal} medium until the A_{600} of the culture approximated 1.2, at which time replicate aliquots of the suspension were incubated for 2 or 3 h, as in the normal assay, with shaking (225 rpm) in the presence of a 1 mM racemic mixture of either *trans*- or *cis*-AEC. Each measurement represents the mean of five replicates. In a similar assay, nontransformed yeast cells grown in YPD medium supplemented with 2% (w/v) D[+]-Gal failed to produce detectable levels of 1-butene when supplied with either of the AEC isomers.

Table II. *Inhibition by Cu^{2+} and Co^{2+} of ethylene production by yeast transformed with pAPY4*

Assay Supplement	Ethylene Production	Relative Rate
	nL of C_2H_4 10^8 cells^{-1} h^{-1}	*% of control*
None	84.4	
Cu^{2+}, 25 μM	25.9[a]	30.7
Co^{2+}, 25 μM	30.9[a]	36.6

[a] *F* test comparison with the control is significant at $P \leq .01$. Transformed yeast cells were cultured in YSM_{gal} medium until the A_{600} of the suspension approximated 1.2. Replicate aliquots were assayed, as described in "Materials and Methods," for ethylene-forming ability in the presence of either 25 μM $CoCl_2$ or $CuSo_4$. Each measurement represents the mean of 10 replicates.

DISCUSSION

The accumulation of mRNA homologous to the cDNA of the plasmid pAP4 has previously been correlated with the temporal synthesis of ethylene during the ripening of, and following the wounding of, preclimacteric apple fruit (Ross et al., 1992). The cDNA sequence has 74% nucleic acid sequence similarity and 88% predicted amino acid sequence similarity to that of the tomato cDNA clone pTOM13 (Slater et al., 1985), which has been shown to encode the ethylene-forming enzyme (Hamilton et al., 1990, 1991). Expression of the pAP4 cDNA sequence in *S. cerevisiae* strain F808− using the expression vector pYES2 (Invitrogen) resulted in the

Table III. *Effect of deleting ascorbate and Fe^{2+} from the ACC oxidase assay medium on the ability of transformed yeast to produce ethylene*

Assay Supplement		Ethylene Production	Relative Rate
Ascorbate	Fe^{2+}		
		nL of C_2H_4 10^8 cells^{-1} h^{-1}	*% of control*
+	+	164.9	
−	+	80.1[a]	48.6
+	−	149.7	90.8
−	−	62.4[a]	37.8

[a] *F* test for comparison with control is significant at $P \leq 0.02$. Transformed yeast cells were cultured in YSM_{gal} medium until the A_{600} of the suspension approximated 1.2. Replicate aliquots of the culture were assayed for the ability of the yeast to convert ACC to ethylene in the presence and absence of supplied ascorbate and/or Fe^{2+}. Where omitted from the assay mixture, the solutions of ascorbate and Fe^{2+} were replaced with an equal volume of sterile distilled water. All other details were as described in the "Materials and Methods." Each measurement represents the mean of 10 replicates.

production of ethylene-forming ability with characteristics similar to those observed in in vivo studies with plant systems (Yang and Hoffman, 1984) and similar to those of ACC oxidase investigated in vitro (Smith et al., 1991; Dilley et al., 1992; Dong et al., 1992a; Kuai and Dilley, 1992).

The ability of the transformed yeast to convert ACC to ethylene depended on the growth phase of the culture in YSM_{gal} medium. In general, although the level of the mRNA corresponding to the cDNA insert of pAP4 continued to increase throughout the exponential growth phase of the transformed yeast, their ethylene-forming ability declined inversely to their growth rate. A relatively high ethylene production rate per 10^8 cells was observed immediately following inoculation of fresh YSM_{gal} medium with a late log phase culture grown in YSM_{glu} medium. This coincided with a lag in the onset of the growth of the culture. The temporal reattainment of maximum ethylene-forming ability also coincided with a slowing in the growth of the culture. Presumably, it is not the level of Gal that is limiting transcription, because the transformed yeast continue to accumulate the mRNA during the growth of the culture in YSM_{gal} medium. Thus, the decline in the ethylene-forming ability of the yeast during their exponential growth may relate more to a redirection of mRNA translation than to the absolute level of the mRNA.

As summarized by Hamilton et al. (1991), the purification of authentic ACC oxidase has been difficult. Until recently this was thought to be due to the difficulty of maintaining an integral membrane association while extracting the enzyme. A number of observations have indicated that there may be some functional requirement of ACC oxidase for membrane integrity (Yang and Hoffman, 1984). This may still hold true. However, recent work in which ACC oxidase has been largely purified (Smith et al., 1991; Dong et al., 1992a; Dupille et al., 1992; Kuai and Dilley, 1992), suggests that the ethylene-forming function of ACC oxidase at least has no requirement for a membrane association. Certainly, all of the cDNA sequences isolated for ACC oxidase so far indicate that the protein is generally hydrophilic and shows no obvious signal peptide that would be associated with the directed targeting of the protein to a specific membrane site. Recent immunocytological studies here have localized ACC oxidase in the cytosol (J. Everard, unpublished data).

The effect of ascorbate and Fe^{2+} on the ability of the transformed yeast to synthesize ethylene is consistent with that found in other studies (Smith et al., 1991; Dilley et al., 1992; Dong et al., 1992a) that indicate the involvement of these components in the overall reaction. According to Dong et al. (1992a), ACC oxidase utilizes equimolar quantities of ACC and ascorbate, with dioxygen and with CO_2 and Fe^{2+} as cofactors, and produces equimolar quantities of ethylene, CO_2, HCN, and dehydroascorbate with the formation of 2 mol of water. The low stimulation of ethylene production observed with supplemental Fe^{2+} may simply result from a nonlimiting growth environment and adequate reserves of Fe^{2+} in the yeast.

The ability to express plant ACC oxidase in yeast makes available a useful system for studying the biochemical regulation of this enzyme. Site-directed mutagenesis studies will be used to elucidate which regions of the protein sequence are involved in the binding of the substrates and cofactors and may provide more information about the reaction mechanism.

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