CLONING OF ACC OXIDASE GENE IN FRUIT OF *LITCHI CHINENSIS* Sonn. AND ITS EXPRESSION IN *E. COLI*

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Abstract

After extraction of total RNA from fruit of litchi cultivar Wuye, the first strand of cDNA was synthesized by reverse transcription with oligo $(dT)_{17}$ as anchored primer. Through comparison of homology among 14 ACC oxidase genes in GenBank, two conserved sites were found and the degenerate primers were synthesized. A 1.2-kb fragment at the 3 end and a 1.1-kb fragment 5 at the end of the ACC oxidase gene were amplified by the RACE approach. The two fragments were cloned into pGEM-T vector and sequenced by Sanger method. The results showed that the ACC oxidase gene of litchi fruit has a 957-bp open reading frame encoding a 36-kD polypeptide with 319 amino acids. The similarities of amino acid sequences between litchi and peach, tomato, carnation, apple, banana, pear and apricot were 82.5%, 70.4%, 73.66%, 74.9%, 78.5%, 75.9%, 77.4%, respectively. Restriction enzyme digestion with *Rsa* I revealed that five members of the ACC oxidase gene family in this cDNA pool. Expression vector pGEMEX-T was constructed and a 60-kD fusion protein including ACC oxidase was induced in *E. coli* by IPTG.

1. Introduction

Ethylene is an endogenous regulator of a variety of stress responses and developmental processes (Abeles *et al*. 1992). Ethylene biosynthesis is strictly regulated, and is induced by a number of different stimuli, including mechanical trauma, pathogen infection, auxins and developmental factors in senescing flowers and ripening fruit (Hamilton *et al*. 1991; Spanu *et al*. 1991; Callahan *et al*. 1992; Pogson *et al*. 1995). The route of ethylene biosynthesis was elucidated by Adams and Yang (Adams *et al*. 1979). In this pathway, characterization of the two main enzymes involved in ethylene biosynthesis, ACC synthase (it catalyses the conversion of S-adenosyl methionine to 1-amino-cyclopropane-1 -carboxylic acid, ACC) and ACC oxidase (it converts ACC to ethylene), led to a better understanding of the molecular basis of the process. Initially it was claimed that ACC synthase plays the major regulatory role since ACC oxidase appears to be constitutively in excess in several tissues (Yang *et al*. 1985). However, the role of ACC oxidase is being reconsidered as ACC oxidase enzyme activity and internal and external factors dramatically affect specific gene expressions (Kende *et al.* 1993). ACC oxidase activity has also been linked to the regulation of physiological events as ethylene autocatalysis (Tang *et al.* 1994).

Ethylene is well known to promote ripening in climacteric fruit. The molecular basis of fruit ripening has been most widely studied in tomato. Mutants blocked in various aspects of fruit ripening are available and numerous ripening-related genes have been described (Callahan *et al.* 1992; Ren *et al*. 1997; Jin *et al.* 1998). In transgenic tomato containing an antisense construction of ACC oxidase, the reduction of ACC oxidase gene expression and the concurrent lower rates of ethylene production appear to be

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physiologically significant since they are closely related to the extent of morphogenetic effects. Moreover, the onset and modulation of developmental processes such as leaf senescence and fruit ripening are affected in ACC oxidase antisense tomato (Hamilton *et al*, 1990) and melon (Ayub *et al*, 1996). In tomato (Bouzayen *et al*, 1993), petunia (Tang *et al*, 1994) and broccoli (Pogson *et al*, 1995), ACC oxidase is encoded by a small multigene family whose expression is tissue-specific and regulated by development and/or hormonal factors, including ethylene.

Litchi is one of important subtropical fruits in China. The fruit has a pleasing appearance and special flavor, but its shelf life is very short. In this study, we aimed to clone the ACC oxidase gene in litchi fruit and express it in *E. coli*. The final objective was to obtain antisense transgenic plants and to prolong the fruit storage life.

2. Materials and methods

2.1. Materials

Litchi (*Litchi chinensis* Sonn.) cultivar Wuye fruit were picked at random from different positions on a bearing tree as they ripened in July, 1998 (Neiliao Orchard, Zhangzhou, Fujian). The fruit samples were held at 25°C for 3 days in the air and then frozen in liquid nitrogen for RNA extraction.

2.2. Methods

2.2.1. RNA extraction

Fruit tissue (1g) was pulverized in liquid nitrogen, quickly transferred into 50-ml tube with 10 mL extraction buffer (25 mmol/L Tris-HCl, pH 8.0, 4 mol/L guanidium chloride, 1% β-mercaptoethanol) and vigorously shaken. The tube was put on ice for 15 min, then 1 mL 2 mol/L NaAc (pH 4.0) and mixed. Added 4mL water-saturated phenol (pH 4.5), 1mL chloroform were added and the tube was vortexed three times. The aqueous phase was separated by centrifugation at 15,000g for 10 min and precipitated RNA was further purified using standard ethanol precipitation.

2.2.2. Reverse transcription

Total RNA(20μg) in reverse transcription buffer (Promega) was primed with oligo $(dT)_{17}$ using AMV RT enzyme (Promega) at 42°C for 2 h. Reverse transcription was stopped by heating at 95 °C for 5 min.

2.2.3. RACE

Rapid amplification of cDNA ends (RACE) was performed according to Frohman (1988). 3'-end amplification of cDNA: The cDNA pool (1μg) and two primers of P_1(5'–CTCT AAAGAANCCCCANTTNCACA- 3') and oligo $(dT)_{17}$ (25 pmol each) in 50 μL of PCR cocktail were denatured (5 min, 95 °C) and heated to 72 °C. Then 2 U of *Taq* DNA polymerase was added. Using a DNA Thermal Cycler (PE-9600), we carried out 40 cycles of amplification by using a step program (94 °C, 1 min; 58 °C 1.5 min; 72°C, 2 min), followed by a 10-min final extension at 72 °C. 5'-end amplification of cDNA: The first strand of cDNA was purified to remove excess dNTPs and anchored primer. For tailing, 1 μL of 6 mM dCTP, 6 μL of 5×tailing buffer and 15 U of terminal deoxy-nucleotidyl transferase (Takara) were added and the mixture was incubated for 10 min at 37 °C and heated for 15 min at 65 °C. The reaction mixture was diluted 10-fold with TE. A 5μL template was used for amplification as described for the 3'-end procedure, except for substitution of the primers P_2 (5'–GGTGGATNACCAATGGNAA NTACAA- 3') and oligo$(dG)_{17}$.

2.2.4. DNA sequencing

Amplified fragments were cloned into pGEM-T vector and sequenced using an ABI Model 373 DNA sequencer with dye-primer and dye-terminator *Taq* sequencing kits. Synthetic oligonucleotide primers were used to obtain overlapping sequences.

2.2.5. Expression of ACC oxidase gene in *E. coli*

Two primers, P_3 (5' –CTGAATTCTGGAGGCTTTCCCAGT- 3') and P_4 (5' –TAGGATCCT CAAATTGGTTGAAATAG- 3'), flanking the open reading frame of the ACC oxidase gene were used to generate a 0.96-kb fragment containing ACC oxidase gene and terminal *Eco* RI and *Bam* HI restriction sites. The PCR reaction was described as earlier. After digestion with *Eco* RI and *Bam* HI, this fragment was ligated to pGEMEX-T, and transformed into *E. coli* DE3. Cells containing the resulting recombinant plasmid (designated p*ACO6*) were cultured in LB medium at 37 ^{o}C to OD_{600}= 0.6, and IPTG was added to a final concentration of 1 mM. After induction with IPTG for 0,0.5, 2, 3, 4 h, cell-free extracts were prepared and subjected to electrophoresis.

3. Results

3.1. Full sequence of ACC oxidase gene by RACE

The two ends of ACC oxidase gene were amplified by RACE. Because both fragments F_1 from the 3'–end and F_2 from the 5'-end included untranslated regions, their accuracy could not be temporarily verified by their masses. Fragment F_1 between P_1 and P_2, was sequenced firstly. Comparisons indicated that it was a homologue of the ACC oxidase gene. This fragment was used as a probe for F_2 and F_3 by Southern blot. F_2 and F_3 showed strong blotting signals, and were confirmed to be parts of ACC oxidase gene. Both of them were sequenced by the Sanger dideoxy method. The whole sequence was obtained by piecing together the sequences of F_2 and F_3. The length of this cDNA was 1170-bp. Starting at the first Met, the predicted open reading frame was 319 amino acids beginning with an ATG at base 37 and ending at base 995 with a poly(A)$^{+}$. Two primers, P_3 and P_4, flanking the open reading frame of ACC oxidase gene were used to generate a 0.96-kb fragment F_4, containing ACC oxidase gene (Fig. 1).

Analyses of the predicted protein indicated its unmodified molecular mass to be 36-kD, with a predicted isoelectric point of 5.4. There is no membrane-associated region. There is one potential N-glycosylation site. The sequence was compared with sequences in GenBank and found to be homologous to other ACC oxidase genes, exhibiting 72.9, 67.7, 72.4, 64.1, 72.1, 73.0 and 75.8% identity to ACC oxidase genes of peach, tomato, carnation, apple, banana, pear and apricot. Sequence similarity has 82.5, 70.4, 73.6, 74.9, 78.5, 75.9 and 77.4% at the amino acid level.

There are three consensus cysteine residues that may indicate a potential disulfide bond, and six consensus histidine residues that may indicate site for ligand binding such as a heme group. There is one consensus glycosylation site. Overall, the sequences exhibit homology throughout the predicted amino acid sequences.

Digestion of the ACC oxidase gene amplification product with *Rsa* I revealed existence of at least 5 members of ACC oxidase gene family of litchi fruit, based on the electrophoretic patterns.

3.2. Expression of ACC oxidase gene from litchi fruit in *E. coli*

A recombinant expression vector p*ACO6* was constructed and transformed into *E. coli* DE3. 1 mM IPTG was added and the bacteria were cultured for 0, 0.5, 2, 3 or 4 h. A 60-kD fusion protein including ACO was induced by IPTG. The arrow in fig. 2 indicates the induced protein.

4. Discussion

Litchi fruit has a very short shelf life. Whether or not it is a climacteric fruit or not is under debate. In several fruits, ACC oxidase mRNA activity rise during ripening when the ethylene burst occurs. According to Tuccker (1993), a control mechanism involving the ACC oxidase activation might be responsible for the transition from system 1 to system 2. ACC oxidase is encoded by a multigene family and there is recent evidence for different ACC oxidase expression patterns (Bouzayen *et al*. 1993). Further work will be performed to elucidate their temporal and spatial expression patterns in litchi. It is clear that ethylene has many effects on different tissues, acting at different developmental stages, and is induced by a range of external stimuli (Abeles *et al*. 1992). The expression patterns of different ACC oxidase genes have, however, just begun to be investigated.

What is the nature of the harvest stimulus that results in increased in ACC oxidase gene expression at litchi fruit harvest? A similar, rapid post-harvest induction of other genes has been reported for asparagus (King *et al*.1992). Harvest disrupts energy, nutrient, and hormone supplies and leads to rapid senescence in fruits. Spanu *et al*. (1991) expressed an RNA related to pTOM13 in *Xenopus laevis* oocytes and obtained EFE (ethylene-forming enzyme) activity. Hamilton *et al*. (1991) also expressed a pTOM13-like clone in yeast and obtained EFE activity; and they obtained decreased ethylene evolution and EFE activity following the insertion of an antisense construct of pTOM13. This supported the idea of the role for pTOM13 in ethylene biosynthesis (Hamilton *et al*. 1990,1991). In our work, p*ACO6* is highly homologous to the pTOM13 family of genes. Understanding how ACC oxidase is regulated and its effect on ethylene levels will help to elucidate the regulation of the many functions that ethylene affects. How this gene is regulated will be the subject of our future research. We plan to insert an antisense construct of the gene into plants to test the ripening response on fruit development and senescence.

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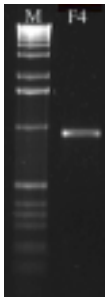
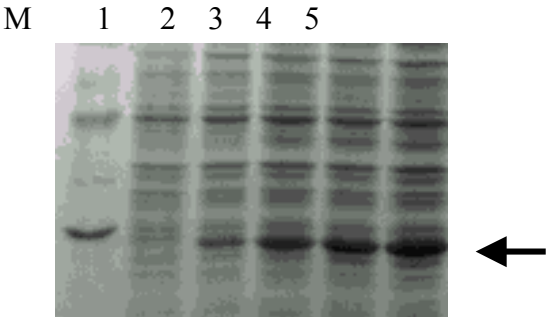
Figures



1. ACC oxidase gene amplified from litchi fruit. F_4: 0.96-kb fragment of ACC oxidase gene; M: 1-kb marker.



2. Protein expression in *E. coli* induced by IPTG.
M: protein marker; Lane 1-5: fusion protein induced for 0, 0.5, 2, 3, 4 h (arrowed).