# Cloning and Expression Analysis of Carboxyltransferase of Acetyl-CoA Carboxylase from *Jatropha curcas*

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A full-length cDNA of the carboxyltransferase (accA) gene of acetyl-coenzym A (acetyl-CoA) carboxylase from *Jatropha curcas* was cloned and sequenced. The gene with an open reading frame (ORF) of 1149 bp encodes a polypeptide of 383 amino acids, with a molecular mass of 41.9 kDa. Utilizing fluorogenic real-time polymerase chain reaction (RT-PCR), the expression levels of the accA gene in leaves and fruits at early, middle and late stages under pH 7.0/8.0 and light/darkness stress were investigated. The expression levels of the accA gene in leaves at early, middle and late stages increased significantly under pH 8.0 stress compared to pH 7.0. Similarly, the expression levels in fruits showed a significant increase under darkness condition compared to the control. Under light stress, the expression levels in the fruits at early, middle and late stages showed the largest fluctuations compared to those of the control. These findings suggested that the expression levels of the accA gene are closely related to the growth conditions and developmental stages in the leaves and fruits of *Jatropha curcas*.

*Key words:* Carboxyltransferase, Fluorogenic Quantitative Real-Time PCR, Overexpression, Physiological Environment

## Introduction

Plant fatty acids are mainly synthesized in plastids, and the prokaryotic form of acetyl-coenzym A (acetyl-CoA) carboxylase (ACCase) regulates the rate of fatty acid synthesis in most plants (Ohlrogge and Browse, 1995) as well as in Yeasts and animals. ACCase is the first enzyme of the fatty acids' biosynthetic pathway. In plants, this enzyme is localized in plastids and is a multisubunit complex composed of multiple copies of three subunits, biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC), and carboxyltransferase ($\alpha$-CT and $\beta$-CT) (Cronan and Waldrop, 2002; Sasaki and Nagano, 2004). ACCases of plants have been reported to be activated by light-dependent changes in the redox status of the chloroplast (Kozaki *et al.*, 2000, 2001). The mechanism of activation is thought to be a thioredoxin-mediated reduction of a disulfide bond as has been reported for a number of other chloroplastic metabolic enzymes. Accumulated evidence showed that the activation of ACCase is caused by light-dependent reduction of carboxyltransferase, but not of BC, via a redox cascade (Sasaki *et al.*, 1997; Hunter and Ohlrogge, 1998; Sellwood *et al.*, 2000). Recent studies showed that the tobacco $\beta$-carboxyltransferase (accD) gene was transformed into plastids causing an increase in the total ACCase level and fatty acid content of the plastids. These findings suggest that the expression of the accD subunit in plastids might limit the total levels of plastidic ACCase and is rate-limiting for fatty acid biosynthesis (Madoka *et al.*, 2002). In addition, early studies have suggested that the carboxyltransferase subunit might be important in the regulating of protein phosphorylation/dephosphorylation (Savage and Ohlrogge, 1999).

*Jatropha curcas* L., commonly known as physic nut, belongs to the family Euphorbiaceae and is today recognized as a petrosubstitute. Various parts of the plant have potent medicinal qualities and considerable commercial value (Debnath and

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Bisen, 2008). Most mechanistic studies of ACCase have focused on the BC component because the gene for the enzyme has been cloned and overexpressed (Shorrosh *et al.*, 1995). Relative few studies have been done on the expression analysis of the carboxyltransferase (accA) gene under physiological stress for more than 20 years despite cloning of these genes (Kozaki *et al.*, 2000; Lee *et al.*, 2004; Nakkaew *et al.*, 2008). Therefore, the present work was conducted in order to isolate and sequence the accA gene of *Jatropha curcas* L. Moreover, the transcriptional levels of the accA gene in leaves and fruits were analyzed at different developmental stages under pH 7.0/8.0 and light/darkness stress.

## Material and Methods

### *Plant materials and growth conditions*

Mature *Jatropha curcas* seeds with the same genetic background were harvested in August 2006 in Panzhihua, Sichuan, China. Seeds were oven-dried, selected and stored in a plastic box (labeled No. 20060822) at 4 °C until use. After saturating in water for about 24–36, they were planted in the garden of, College of Life Science, Sichuan University, China. The whole garden was divided into four plots: namely groups A, B, C, and D. They were cultured under pH 7.0/8.0 conditions and light/darkness stress, respectively.

Seed germination and sprouting of the main leaves were regarded as the early growth stage, and plucking of the leaves, three months after the main leaves have sprouted; was regarded as the middle growth stage. After six months, the plants entered the lowering period which was regarded as the late growth stage. Specimens were collected from the four plots (A, B, C, D) in line with the three growth stages which were logically classified into twelve groups. Under suitable water and fertilizer conditions, seedling plant cuttings go into the reproductive growth stage faster than usual seedling plants cuttings were made in April 2006. Selected stems of *Jatropha curcas* from Panzhihua, Sichuan were cut and planted in the garden.

At the same time, the plantlets were treated in the same way as the leaves at pH 7.0/8.0 and under light/darkness conditions. The flowering period started in September of the same year and the flowers bloomed about 5 d. Upon the completion of pollination, the fruit began to develop while the sepal and the petals of the female flowers continued to grow to a certain degree in order to protect the developing fruit. The fruits were fully developed three months after pollination. The formation of the fruits went through enlargement, dehydration, and decolouration – from green to yellow and from yellow to brown. Young fruits appeared 2–3 d after the female flowers have been pollinated. The early stage of the fruits was a 20-d period commencing from the completion of the pollination. The middle stage of the fruit began 20 d after the commencement of the early stage. The ripening of the fruits – yellowing from green, which is the late stage of the fruits – was 20 d after the commencement of the middle stage. During the experiment, the plants were divided into four groups according to the different stress conditions. The experiments were repeated three times.

### *RNA isolation, PCR cloning and sequencing*

Total RNA was extracted by RNA extraction kit (Watson Biotechnologies, Shanghai, China). PrimeScript™ 1st strand cDNA synthesis kit was used for cDNA synthesis (TaKaRa Biotechnology Co. Ltd., Dalian, Liaoning, China). In the first round of the cloning experiments, combinations of primers designed for amplification of a fragment of the plastid accA gene were used. The amplifications were carried out with the TaKaRa One Tube real-time polymerase chain reaction (RT-PCR) system according to the manufacturer's instructions, and a Perkin-Elmer 2400 apparatus was used under the following conditions: 95 °C for 5 min, 94 °C for 2 min, 57 °C for 90 s, 72 °C for 90 s (10 cycles), followed by 25 cycles carried out under the same conditions with the exception that the extension step (3 min) contained an auto-extension time of 5 s per each cycle and the final extension step was at 68 °C for 7 min.

The high-fidelity ExTaq DNA polymerase (TaKaRa Biotechnology Co. Ltd.) was used for PCR amplification. The size of the amplification products was estimated following electrophoresis on 1.0% agarose gels. The amplified DNA products were purified using the EZNA™ gel extraction kit (Omega). The purified PCR products were cloned into a pMD18-T vector (TaKaRa Biotechnology Co. Ltd.) according to the manufacturer's instruction. The cloned PCR products were sequenced, which was performed by TaKaRa Biotechnology Co. Ltd. All the sequences used in

the expression analysis were derived from at least three independent clones.

*Fluorogenic quantitative RT-PCR*

In order to identify the expression levels of the accA gene at different developmental stages and under stress conditions in the leaves and fruits of *Jatropha curcas*, primers for quantification by RT-PCR were designed. PCR amplifications were carried out with the specific primers as describe in Table I, including 18 s1, 18 s2, Tar 1, and Tar 2, respectively. cDNA synthesis was conducted as described previously. The cycler iQTM Multi-Wavelength Real-Time Fluorescent PCR system (Bio-RAD, USA) was used to amplify and measure the prepared standard samples and cDNA sample template for standard quantitative analysis. 18s rRNA of *Jatropha curcas*, used as inner control, was amplified in parallel with the target gene allowing gene normalization and providing the certification. Detections of RT-PCR products were carried out by the SYBR Green I fluorescence dye. Experiments chose 18s rRNA as the house-keeping gene and went through the reaction system with ingredients as follows: 12.5 $\mu$l 2×SYBR Premix ExTaq TM with a total volume of 20 $\mu$l, 0.5 $\mu$l SYBR Green I (10,000-fold dilution), 0.5 $\mu$l of each 10 $\mu$M primers, 0.5 $\mu$l cDNA (the equivalent of 20 ng total RNA) as template and sufficient DEPC-treated water. The fluorescent gathering was carried out in the third step of every cycle. The solution was subsequently denatured at 95 °C for 30 s, annealed at 55 °C for 1 min with an increment of 0.5 °C every 10 s. The fluorescence value was measured during the processes.

*Statistical analysis*

All values shown in this paper are the mean of at least three replicated treatments for each condition. Data tested at significant levels of $P$ value were less than 0.05 using one-way ANOVA.

## Results and Discussion

ACCase catalyzes the formation of malonyl-CoA from acetyl-CoA and bicarbonate in the first committed step of *de novo* fatty acid synthesis. The enzyme transcarboxylase (TC) catalyzes an unusual reaction, and transfers a carboxylate group from methylmalonyl-CoA to pyruvate to form oxaloacetate and propionyl-CoA (Sasaki and Nagano, 2004). In order to better characterize *Jatropha curcas* carboxyltransferase, we isolated a full-length cDNA from *Jatropha curcas* and two oligonucleotides designed on the basis of the conservation sequence of some plant carboxyltransferases. Fig. 1 shows that a cDNA fragment of about 520 bp was first obtained by PCR. Based on the conservation sequence of some plant carboxyltransferases, we obtained about 480-bp 3′-terminal fragments and about 600-bp 5′-terminal fragments by cloning. Based on a pair of primers of Tar time 1 and Tar time 2 (Table I), about 1200-bp fragments were amplified and obtained, with cDNA as templates. Sequence data from this article have been deposited at NCBI under accession number EF095236. The *Jatropha curcas* carboxyltransferase cDNA consists of 1149 bp encoding a polypeptide of 383 amino acids with a calculated molecular mass of 41876.2 Da and a predicted isoelectric point of 9.01 (Fig. 2).

The transcription levels of the accA gene in the leaves and fruits at the early, middle and late developmental stages under pH 7.0 and 8.0 stress are shown in Table II. The expression levels of the accA gene in the leaves at the middle and late stages increased by about 24% and 8% under

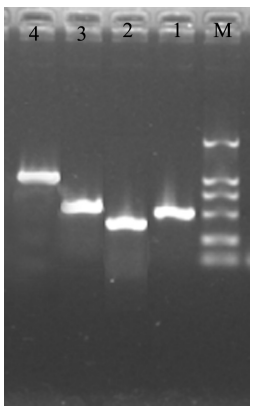


Fig. 1. Amplification fragments of the carboxyltransferase cDNA. Lane 1, PCR reaction using Upz and Doz PCR primers resulting in the 520-bp fragment; lane 2, 5′-RACE reaction with Race primer producing the 480-bp fragment; lane 3, 3′-RACE reaction with Oligod(T)18 primer resulting in the 600-bp fragment; lane 4, PCR reaction using the Tar time 1 and Tar time 2 primers leading to the 1152-bp full-length cDNA fragment; lane M, marker (DL 2000), from top to bottom were 2000, 1000, 750, 500, 200, and 100 bp, respectively.

Table I. Primer sets used for PCR and RT-PCR.

| Primer name | Primer sequence (5′→3′) | Length [bp] |
|---|---|---|
| Upz[a] | GTGCGGAAGATGGCAAATGA | 20 |
| Doz[a] | CAATCGACACAATTGGTACC | 20 |
| Up3[a] | CACACGTTGTATCGGGGTCA | 20 |
| Do5[a] | CTGAAGAACTAGGCCAGGGT | 20 |
| Oligod(T)18[a] | GCTGTCAACGATACGCTACGTAACGGCATGACAGTG (T)18 | 54 |
| Race[a] | GTCAA CGATACGCTACGTAACG | 22 |
| Tar time 1[a] | CGGTCGACATGGCTTCTATATC | 22 |
| Tar time 2[a] | CGATTGCGGAATTCCTATGTC | 21 |
| 18 s1[b] | ACGGGTGACGGAGAATTAGG | 20 |
| 18 s2[b] | TACCAGACTCATTGAGCCCG | 20 |
| Tar 1[b] | GGCCAGGGTGAAGCAATTGC | 20 |
| Tar 2[b] | GAGAAGCTTTGGCACTCTTC | 20 |
| 18 s rRNA Rtime 1[b] | GTGTAAGTATGAACTAATTC | 20 |
| 18 s rRNA Rtime 2[b] | GCCAAGGCTATAAACTCGTTGA | 22 |

[a] Specific primers for gene cloning. [b] Gene-specific primers for expression analysis.

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1    ATG GCT TCT ATA TCG CAT TCC CCA TCT GCA TTT CGT GGA ACT TCA GCT TCA GAT CTT CTT
1      M   A   S   I   S   H   S   P   S   A   F   R   G   T   S   A   S   D   L   L
61   AGG AGC TCC AGC AAT GGA GTT TCT GGT ATC CCT TTG AGG ACA TTG GGA AGA GCG CGA TTT
21     R   S   S   S   N   G   V   S   G   I   P   L   R   T   L   G   R   A   R   F
121  AGC TCC AAG ACG AAG GAT TTT GCA GTG ATT GCT GAG ATT AGG AAG GTG AAG AAG CAA GAA
41     S   S   K   T   K   D   F   A   V   I   A   E   I   R   K   V   K   K   Q   E
181  TAT CCT TGG CCA GAT AAT CCG GAT CCA AAT GTA GCA GGA GGA GTG CTC TCT CAT CTT TCG
61     Y   P   W   P   D   N   P   D   P   N   V   A   G   G   V   L   S   H   L   S
241  CCT TTT AAG CCT TTA AAA GAG AAG CCA AAG CCA GTT ACT TTG GAT TTT GAA AAG CCA CTT
81     P   F   K   P   L   K   E   K   P   K   P   V   T   L   D   F   E   K   P   L
301  ATT GGT TTA GAG AAG AAG ATT ATC GAT GTG CGG AAG ATG GCA AAT GAA ACT GGT TTA GAC
101    I   G   L   E   K   K   I   I   D   V   R   K   M   A   N   E   T   G   L   D
361  TTC ACT GAT CAG ATT ATT TCA CTA GAG AAT AAA TAT CAA CAG GCT CTA AAG GAT TTG TAT
121    F   T   D   Q   I   I   S   L   E   N   K   Y   Q   Q   A   L   K   D   L   Y
421  ACA CAT TTG ACC CCG ATA CAA CGT GTG AAT ATT GCA CGG CAC CCT AAC AGG CCC ACT TTC
141    T   H   L   T   P   I   Q   R   V   N   I   A   R   H   P   N   R   P   T   F
481  CTT GAT CAT GTA TTT AGC ATT ACC GAT AAG TTT GTG GAA CTT CAC GGA GAT CGA GCG GGT
161    L   D   H   V   F   S   I   T   D   K   F   V   E   L   H   G   D   R   A   G
541  TAT GAT GAT CCT GCT ATC GTT ACT GGT ATA GGA ACC ATA GAT GGT AGA AGA TAC ATG TTC
181    Y   D   D   P   A   I   V   T   G   I   G   T   I   D   G   R   R   Y   M   F
601  ATG GGT CAC CAG AAG GGT AGA AAC ACC AAA GAG AAC ATT ATG CGC AAT TTT GGG ATG CCT
201    M   G   H   Q   K   G   R   N   T   K   E   N   I   M   R   N   F   G   M   P
661  ACT CCC CAC GGA TAC CGG AAG GCT CTA AGG ATG ATG TAT TAC GCA GAC CAC CAT GGA TTT
221    T   P   H   G   Y   R   K   A   L   R   M   M   Y   Y   A   D   H   H   G   F
721  CCT ATA GTT ACT TTT ATT GAC ACA CCC GGG GCA TTT GCA GAC CTC AAA TCT GAA GAA CTA
241    P   I   V   T   F   I   D   T   P   G   A   F   A   D   L   K   S   E   E   L
781  GGC CAG GGT GAA GCA ATT GCC CAT AAT TTG AGG ACA ATG TTT GGT TTA AAG GTA CCA ATT
261    G   Q   G   E   A   I   A   H   N   L   R   T   M   F   G   L   K   V   P   I
841  GTG TCG ATT GTT ATT GGG GAA GGT GGC TCT GGT GGT GCT CTG GCC ATT GGC TGT GCT AAT
281    V   S   I   V   I   G   E   G   G   S   G   G   A   L   A   I   G   C   A   N
901  AAA TTG TTA ATG CTT GAA AAT GCA GTT TTC TAT GTT GCC AGT CCT GAA GCA TGT GCA GCA
301    K   L   L   M   L   E   N   A   V   F   Y   V   A   S   P   E   A   C   A   A
961  ATT TTA TGG AAG AGT GCC AAA GCT TCT CCA AAG GCG GCT GAA AAA TTG AAG ATC ACT GCT
321    I   L   W   K   S   A   K   A   S   P   K   A   A   E   K   L   K   I   T   A
1021 ACT GAG TTG TGC ATG TTG CAA ATT GCA GAT GGT GTC ATT CCT GAG CCA TTA GGT GGC GCA
341    T   E   L   C   M   L   Q   I   A   D   G   V   I   P   E   P   L   G   G   A
1081 CAT GCA GAT CCA TCA TGG ACC TCA CAA CAG ATA AAG AAT GCA ATT AAG GAA TCT ATG GAT
361    H   A   D   P   S   W   T   S   Q   Q   I   K   N   A   I   K   E   S   M   D
1141 GAG CTG ACA TAG
381    E   L   T   *
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Fig. 2. The nucleotide sequence and deduced amino acid sequences of the *Jatropha curcas* carboxyltransferase cDNA. The sequences are numbered at the left. * represents the stop codon.

Table II. Expression levels of the carboxyltransferase gene in leaves and fruits of different developmental stages (early, middle and late) under pH stress.

| pH | Tissue | Developmental stage | | |
|---|---|---|---|---|
| | | Early | Middle | Late |
| 7.0 | Leaves | 1 ± 0.03 | 1.24 ± 0.05 | 1.08 ± 0.04 |
| 8.0 | Leaves | 5.5 ± 0.22 | 5.94 ± 0.24 | 8.46 ± 0.31 |
| 7.0 | Fruits | 5.03 ± 0.19 | 8.63 ± 0.31 | 6.11 ± 0.27 |
| 8.0 | Fruits | 47.3 ± 1.78 | 54.4 ± 2.17 | 35.9 ± 1.16 |

Expression levels of the carboxyltransferase gene in the early stage leaves were defined as control, and 18s rRNA was used as house-keeping gene. Based on control and reference, the relative copy numbers of other data are computed. Data were analyzed by using the $2^{-\Delta\Delta CT}$ method. The values given are the averages of three experiments.

pH 7.0 stress compared to the control, respectively. However, the expression levels in the leaves increased significantly under pH 8.0 stress compared to the control, and the levels at the early, middle and late stages were about 5.5, 5.94 and 8.46 times that of the control, respectively. These results suggested that the expression levels in the leaves at the same developmental stage but treated at pH 7.0 and pH 8.0 are significantly different. In the fruits, the expression levels at the early, middle and late stages under pH 7.0 stress were about 5.03, 8.63 and 6.11 times that of the control. However, the expression levels increased significantly under pH 8.0 stress, representing about 47.3, 54.4, and 35.9 times that of the control, respectively. Our findings suggested that the expression levels in the fruits at the same developmental stage are significantly higher than that in the leaves under pH 7.0/8.0 stress. Previous studies suggested that over-expression of the $\beta$-carboxyltransferase (accD) gene resulted in an increase in the ACCase levels in tobacco. The level of the accD subunit is a determinant of final ACCase levels, and this final enzyme level is in part controlled post-transcriptionally at the level of subunit assembly. The resultant transformants grew normally and the fatty acid content was significantly increased in leaves. The transformants displayed extended leaf longevity and had a two-fold increase in the seed yield over the control value, eventually almost doubling the fatty acid production per transformed plant relative to the control and wild-type plants (Madoka *et al.*, 2002; Nakkaew *et al.*, 2008). Based on the above results, our finding suggested that the expression levels of the accA gene in the leaves and fruits shows a clear correlation with the developmental stages, pH values and organ types. In addition, there might be a correlation between the increasing expression levels of the accA gene and the regulation of the ACCase levels. However, the expression levels of the accA gene are complex and need further study.

The expression levels of the accA gene in leaves and fruits at different developmental stages (early, middle, and late) under light and darkness stress are shown in Table III. Under darkness stress, the expression levels of the accA gene in leaves at the middle and late stages increased by about 1.15- and 1.13-fold compared to the control, respectively. However, the expression levels in leaves at the early, middle and late stages under light stress were 71.5, 93.1 and 85 times that of the control, respectively. In fruits under darkness stress, the expression levels at the early, middle

Table III. Expression levels of the carboxyltransferase gene in leaves and fruits of different developmental stages (early, middle and late) under darkness/light stress.

| Condition | Tissue | Developmental stage | | |
|---|---|---|---|---|
| | | Early | Middle | Late |
| Darkness | Leaves | 1 ± 0.03 | 1.15 ± 0.04 | 1.13 ± 0.04 |
| Light | Leaves | 71.5 ± 2.58 | 93.1 ± 3.65 | 85.04 ± 3.23 |
| Darkness | Fruits | 10.7 ± 0.46 | 12 ± 0.49 | 8.4 ± 0.33 |
| Light | Fruits | 2005.2 ± 89.8 | 2468.7 ± 109.1 | 1745.6 ± 68.6 |

Expression levels of the carboxyltransferase gene in early stage leaves were defined as control, and 18s rRNA was used as house-keeping gene. Based on control and reference, the relative copy numbers of other data are computed. Data were analyzed by using the $2^{-\Delta\Delta CT}$ method. The values given are the averages of three experiments.

and late stages were 10.7, 12 and 8.4 times that of the control. However, the expression levels at the early, middle and late stages under light stress were 2005.2, 2468.7 and 1745.6 times that of the control, respectively. In addition, the expression levels in the fruits at the early, middle and late stages were 187.4, 205.7 and 207.8 times under light stress those of the darkness stress, respectively.

Activation of fatty acid synthesis by light is caused partly by activation of the regulatory enzyme ACCase. Some reports suggested that ACCase is primarily activated through two physiological processes. Process one involves the reaction of the CT subunit on the BC subunit and BCCP subunit. Process two involves the disulfide bond in the cysteine residue in the CT subunit. The disulfide bond is reduced by photoinduction and contributes to the activation of ACCase (Sasaki *et al*., 1995; Gengenbach, 2004). Our findings suggested that the expression levels of the accA gene in leaves and fruits were very high under light stress compared to those under darkness stress.

These findings suggest that the expression levels of the accA gene are dependent upon the physiological environment and developmental stage in the leaves and fruits of *Jatropha curcas*. Significant increases in the expression levels of the accA gene suggested that it might play a pivotal transitional role in the catalytic activity of ACCase and, ultimately, determines the fatty acid synthesis. Although the mechanism of accA gene regulation needs further elucidation, these findings offer a potential method for raising the plant productivity and oil production.

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