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Effects of N-terminal deletions on 1-aminocyclopropane-1-carboxylate synthase activity

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Abstract **A series of nested N-terminal deletions were made on the full-length (*wt*) and C-terminal deleted (Cdel) 1-aminocyclopropane-1-carboxylate synthase cDNAs. These *wt* and mutant ACC synthases were over-expressed in a heterologous *E. coli* expression system. It was found that removal of an amino acid region (residues 2–12) from the non-conserved N-termini of *wt* and Cdel ACC synthases led to a slight increase in both in vivo ACC production and in vitro ACC synthase activity. Further deletion of 11 amino acids through Glu-23 from the N-termini of both *wt* and Cdel ACC synthases resulted in a substantial reduction in both in vivo ACC production and in vitro enzyme activity. Deletion of an amino acid region, residues 3 through 27, from the N-terminus of ACC synthase abolished enzyme activity completely. Kinetic analysis of a highly purified double-deletion mutant (NCdel-1) of ACC synthase demonstrated that the K_m of this mutant is 42 μM, which is much smaller than that of the corresponding Cdel (280 μM) and closer to that of *wt* (22 μM) reported previously, suggesting a clear effect of the non-conserved N-terminal region on its ACC synthase function.**

Key words: Tomato fruit; ACC synthase; Amino-terminus; Deletion; Kinetics

1. Introduction

The biosynthesis of the plant hormone ethylene in higher plants is controlled by an unique plant enzyme, ACC synthase (EC 4.4.1.14), which catalyzes the conversion of *S*-adenosylmethionine (AdoMet) to ACC, the immediate precursor of ethylene [1]. This phytohormone has a profound and versatile effect on plant growth and development, especially on fruit ripening and flower fading. In recent years, the purification of this enzyme and the cloning of genes encoding this enzyme have been the focus of ethylene biology. Monoclonal antibodies against a wound-inducible tomato ACC synthase were first raised in 1986 [2]. cDNAs encoding ACC synthases were later isolated by screening a cDNA library using either polyclonal antibodies [3,4] or degenerated oligonucleotides [5,6]. To date, ACC synthase cDNAs have been isolated from many crops of both agricultural and horticultural importance [7,8].

On optimum alignment of ACC synthase amino acid sequences, it was found that there were seven highly conserved regions [6] and 16 invariant amino acid residues [9] in this enzyme. The overall amino acid sequences are 72–81% similar and 53–67% identical with the exception of the amino- and, particularly, the carboxyl-termini of ACC synthase which were found to be highly variable in both length and primary sequence [10]. Expression of the full-length polypeptides of apple, zucchini, tomato and squash ACC synthases in a heterologous *E. coli* system has produced a functional enzyme. The molecular weights of these full-length polypeptides, as determined by SDS-PAGE analysis, are 52, 53, 54 and 60 kDa, respectively [4,9,10,11]. They are 4–10 kDa larger than the same enzyme directly isolated from plant tissue [2,11,12,13,14]. This size difference observed suggests that the proteolytic processing might have occurred in vitro during the isolation of the native ACC synthase from plant tissues. However, the existence of a 47 kDa ACC synthase has been shown by in vivo labeling of the wounded tomato fruit pericarp tissue followed by immunoprecipitation and SDS-PAGE analysis [15], suggesting that the proteolytic processing may also occur in vivo. Thus, ACC synthase gene expression may also be controlled at the post-translational level.

It has been demonstrated previously that the non-conserved C-terminus affects enzyme function and conformation [10]. Deletion of 46 amino acids at the C-terminus of a wound-inducible ACC synthase increased both V_{max} and K_m of this enzyme. Deletion of 52 amino acids resulted in a 46 kDa enzyme with increased V_{max} and decreased K_m. In this experiment, we investigated the effect of N-terminal deletion on enzyme function, and delimited the particular amino acid or amino acid region at the N-terminus which is essential for a minimum enzymatic function or for enhanced enzyme activity.

2. Materials and methods

2.1. Bacteria strains and vectors

INVαF′: *end*A1, *rec*A1, *hsd*R17 (r_k^-, m_k^+), *sup*E44[$^-$, *thi*1, *gyr*A, *rel*A1, F80*lac*Z DM15D, (*lac*ZYA-*arg*F)U169, *deo*R$^+$F′ (Invitrogen, The Netherlands); BL21(DE3)pLysS: F$^-$, *omp*T, *hsd*S$_B$(r_B^- m_B^-), *dcm*, *gal*, (DE3) pLysS, Cm^r (Novagen, Madison, WI); pET11d vector (Novagen, Madison, WI).

2.2. Oligonucleotides

Primers used in PCR and DNA sequencing were synthesized using a DNA synthesizer, Gene Assembler (Pharmacia, Uppsala, Sweden). P_N and P_C stand for N-terminal and C-terminal primers, respectively. They were employed to synthesize and amplify by PCR the *wt* ACC synthase (LE-ACS2) cDNA from a tomato fruit specific cDNA library, ripening UC82-B (Stratagene, La Jolla, CA). The rest nine primers used to direct the synthesis of mutant ACC synthases were designed based on the known ACC synthase cDNA sequence (LE-ACS2). Table 1 lists the eleven oligonucleotides.

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Abbreviations: aa, amino acid; ACC, 1-aminocyclopropane-1-carboxylic acid; AdoMet, *S*-adenosylmethionine; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid: PLP, pyridoxyl 5′-phosphate; IPTG, isopropyl-β-D-thiogalactopyranoside; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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2.3. Construction of pET11d-ACS recombinant plasmids

The *wt* and mutant ACC synthase cDNAs were synthesized and amplified by PCR with 5 μl of phage solution (10^9 pfu) in accordance with the manufacturers' protocol for 35 cycles at 94°C (1.2 min), 55°C (1.5 min) and 72°C (2.5 min) [16]. The PCR products were then double-digested by *NcoI*/*Bam*HI restriction enzymes and fractionated on low melting point agarose gel (1%) to separate the digested DNA from phage DNA and primers. The ACC synthase cDNA fragments were extracted from the excised gel slices by Geneclean Kit (Bio 101 Inc., La Jolla, CA) and ligated to the *NcoI*/*Bam*HI double-digested 5.7 kb pET11d vector (Novagen, Madison, WI). The pET11d-ACS chimeric plasmids were transformed into the competent INVαF′ cells. DNA sequencing was carried out using these chimeric plasmids. Those containing the correct sequence were identified and retransformed into an *E. coli* host, BL21(DE3)pLysS. This strain was specially designed to over-express a foreign protein by the inclusion of T7 RNA polymerase [17] and the proteolytic processing of the expressed foreign protein is minimized due to the absence of soluble and membrane proteases.

2.4. Over-expression of wt and mutant ACC synthases in E. coli

BL21(DE3)pLysS cells harboring a pET11d-ACS recombinant plasmid were grown on a TBM9 plate in the presence of 100 μg/ml ampicillin and 35 μg/ml chloramphenical. TBM9 medium (5 ml) containing 150 μg/ml of ampicillin was inoculated with a single colony and incubated at 37°C overnight with constant shaking. The overnight culture (0.5 ml) was transferred into 50 ml of TBM9 medium. Cells were grown at 37°C with constant shaking. When the cell density reached OD_{600} of 0.5, IPTG was added to the cell culture to a concentration of 0.4 mM. The culture was then transferred to 25°C for an hour before the addition of rifampicin (200 μg/ml). The induction continued for 2 more hours at 25°C with constant shaking. The cells were harvested by centrifugation at 5000 × *g* for 10 min at 4°C. Both supernatant and cell pellet were stored at −70°C for later use.

2.5. Preparation of ACC synthase extract

The frozen cells were resuspended in 2.5 ml of extraction buffer containing 50 mM EPPS (pH 7.5), 10 μM PLP, 5 mM EDTA, 2 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml pepstatin and 10 μg/ml leupeptin [8]. Cells were lysed by sonication on ice and centrifuged for 20 mins at 15,000 × *g* at 4°C to remove cell debris. The 2.5 ml cell extract obtained was mixed with polyethyleneimine solution to a final concentration of 0.4%, incubated on ice for 20 min, and then centrifuged for 20 min at 15,000 × *g* at 4°C. The supernatant was fractionated on a 10 ml Sephadex G-25 column with the diluted ACC synthase extraction buffer (0.05 ×) as the mobile phase. The first 2 ml of eluate after the void volume was collected and frozen at −70°C for later use.

2.6. Purification of the NCdel-1 ACC synthase mutant

The frozen cell pellet collected from 5 liters of IPTG-induced pET-ACS:BL21(DE3) plysS cell culture was resuspended in 100 ml of extraction buffer containing 20 mM EPPS (pH 8.3), 1 mM DTT, 1 mM EDTA, 10 μM PLP, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin and 0.1 mM PMSF followed by sonication. The supernatant was subject to fractionation by ammonium sulfate. The fraction precipitated between 35% and 79% saturation was resuspended in 10 ml of extraction buffer and desalted by dialyzing against the same buffer overnight. The dialyzed enzyme extract was applied in four 2.5 ml aliquots to a DEAE 10HR column and eluted with a gradient of 20–400 mM NaCl at a flow rate of 1 ml/min. Each fraction was assayed for ACC synthase activity and those containing peak activity were pooled and concentrated to 1.5 ml using an Amicon Centricon 10 concentrator. After dialysis against gel filtration buffer containing 50 mM K_2HPO_4 (pH 7), 150 mM NaCl, 2 mM DTT, 5 μM PLP, 0.25 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 0.1 mM PMSF, the concentrate was loaded in six 0.25 ml aliquots onto two-consecutively linked Superose-6 and -12 columns and eluted at a flow rate of 0.25 ml/min. Fractions containing peak activity were pooled and concentrated to 1.2 ml using the same concentrator. The resulting enzyme extract was used for the study of the enzyme kinetics of ACC synthase deletion mutant.

2.7. Determination of ACC content, ACC synthase activity and kinetics

Aliquots of TBM9 medium of 100 μl or 500 μl from the IPTG-induced pET11d-ACS: BL21(DE3)pLysS cell culture were mixed with water to make a final volume of 500 μl. The amount of ACC in this 0.5 ml of medium was determined by the method of Lizada and Yang [18].

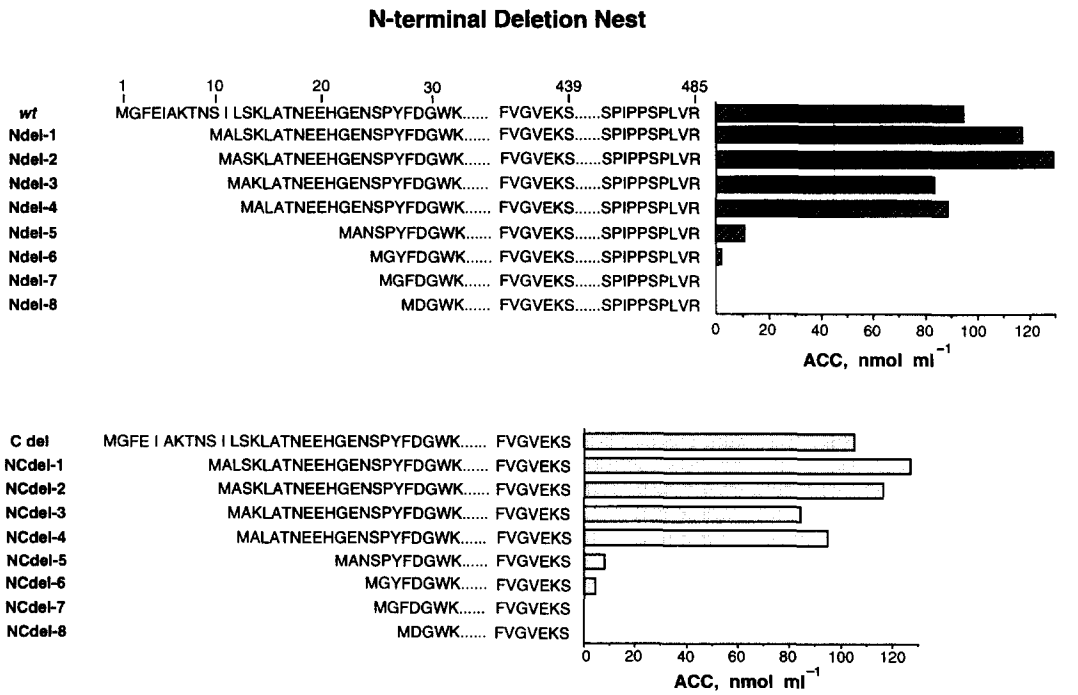

Fig. 1. In vivo ACC production by *E. coli* carrying *wt* and mutant ACC synthase genes. The full-length wound-inducible tomato fruit ACC synthase (*wt*), the N-terminal deletion mutants (Ndel-1, -2, -3, -4, -5, -6, -7, -8), the C-terminal deletion mutant (Cdel) and the double-deletion mutants (NCdel-1, -2, -3, -4, -5, -6, -7, -8) were generated by PCR using oligonucleotides listed in Table 1 as primers for initiation of DNA polymerization. The dots represent the amino acids sequence omitted. The residues are numbered according to the *wt* ACC synthase amino acid sequence. The hatched and shaded bars represent the level of ACC accumulated in *E. coli* growth medium after inoculation. Each bar represents an average of two or three values obtained from two or three independent isolates. The conditions used to induce ACC synthase expression in *E. coli* are described in section 2.

The AdoMet used in the enzyme assay which contained 59% of (S,S) diastereomer form [9] was purchased from Boerhinger-Mannheim Biochem (Germany). ACC synthase activity was assayed by incubating the crude (5–15 μg) or the highly purified (0.3 μg) ACC synthase extract with AdoMet at 30°C for 30 min in a reaction buffer containing 25 mM HEPES at pH 8.5, 10 μM PLP and 1 mM DTT. One unit of ACC synthase activity is defined as 1 nmol of ACC formed per hour at 30°C. The kinetic parameters, K_m and V_{max} of *wt* and deletion-mutated ACC synthases were determined from the Michaelis-Menten equation using a computer program ENZFITER (Biosoft, Cambridge, UK). The protein concentration in each assay was determined by the Bradford method [19].

3. Results and discussion

Alignment of many ACC synthase primary sequences has shown that the core ACC synthase amino acid sequence is highly conserved except at the two termini, which are hypervariable. Previously, it was shown that elimination of 46–52 aa at the C-terminus from the full-length dimeric enzyme resulted in an enzymatically more active monomer [10]. Deletion of 57 aa, including Arg-429, from the C-terminus of the wound-inducible tomato fruit ACC synthase abolished its activity [10]. However, the role of the N-terminal region in ACC synthase function was not studied.

To further explore the role of the N-terminus in the ACC synthase function, eight N-terminal deletion mutants (Ndel series), one C-terminal deletion mutant (Cdel) and eight double-deletion mutants (NCdel series) were constructed using PCR methodology (see Fig. 1). In the process of N-terminal deletion, methionine has to be retained to ensure the initiation of translation. An additional amino acid immediately following methionine was inserted to create a *Nco*I restriction enzyme recognition site for the convenience of subcloning into the expression plasmid. In Ndel-1 to -5 and NCdel-1 to -5 mutants, alanine was introduced, whereas in Ndel-6, -7 and NCdel-6, -7 mutants, glycine was introduced. In Ndel-8 and NCdel-8 mutants, only methionine was introduced in front of Asp-29. These N-terminal deletion mutants (Ndel series) as well as double deletion mutants (NCdel series) were constructed into the expression vector pET11d (Novagen, Madison, WI). Two or three isolates of each construct were obtained. Since the substrate AdoMet is endogenous to and highly prevalent in *E. coli* cells, ACC synthase over-expressed in BL21(DE3)pLysS cell could continue to convert the endogenous AdoMet to ACC. ACC thus produced is subsequently secreted into the growth medium. Assuming that the ACC accumulation in growth medium is directly related to the ACC synthase activity in these mutants, we have assayed the ACC accumulated in the growth medium after inoculation. The ACC concentration produced by *wt*, Ndel-1, -2, -3, -4 and NCdel-1, -2, -3, -4 clones varies from 83 to 128 nmol per ml (Fig. 1). NCdel-1, -2 or Ndel -1, -2 produced a higher amount of ACC in the growth medium than that of wild type, suggesting an enhanced enzyme activity or a higher level of over-expression associated with the deletion mutant Ndel-1, -2 and NCdel-1, -2. Each value shown in Fig. 1 represents an average value of two or three different isolates induced under the same conditions. A dramatic reduction in ACC content was observed in Ndel-5, and -6 mutants where the ACC concentration in the growth medium was only 5–11 nmol per ml. A similar trend was also found in NCdel-5, and -6 mutants. Further deletion through Tyr-27 resulted in a complete loss of ACC accumulation as demonstrated by Ndel-7 and -8 and NCdel-7 and -8 (Fig. 1).

To confirm this finding, the enzyme activity of the ACC synthase was examined with an AdoMet concentration of 180 μM. Similarly, a significant increase in enzyme activity was observed when the deletion progressed through Leu-12 as demonstrated by Ndel-1, -2 and NCdel-1, -2 shown in Fig. 2. However, when Ser-13 was removed, the enzyme activities of Ndel-3 and NCdel-3 decreased to 35% and 8% of the *wt* and Cdel control, respectively. These results are at variation with that of ACC content assay, where a sharp decrease in ACC accumulation in Ndel-3, -4 and NCdel-3 and -4 mutants was not observed. Two explanations can be offered: first, the mutant enzymes (Ndel-3, -4 and NCdel-3, -4) lacking Ser-13 is so unstable that they lose their enzyme activities during the preparation of

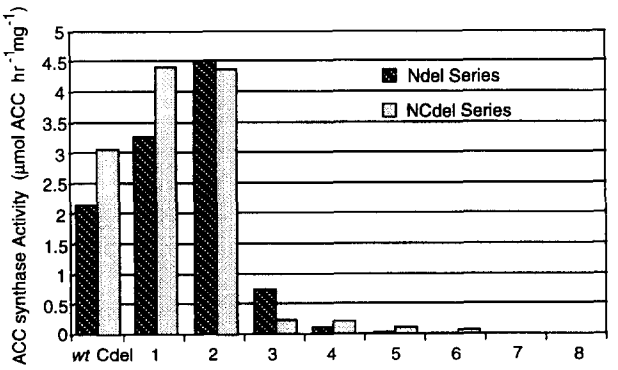

Fig. 2. The enzyme activity of *wt* and mutant ACC synthases. The *wt* and mutant ACC synthases over-expressed in *E. coli* were purified 4.4-fold by polyethyleneimine precipitation and Sephadex G-25 column and assayed under conditions described in section 2. The hatched bars represent the enzyme activity of *wt* ACC synthase and its N-terminal deletion mutants. The shaded bars represent the enzyme activity of C-terminal deletion mutants and double deletion mutants. The protein concentration was determined by the Bradford method. Aliquots of 5–15 μg of ACC synthase were used in enzyme assay. Each value represents an average of at least two values obtained from two or three independent isolates.

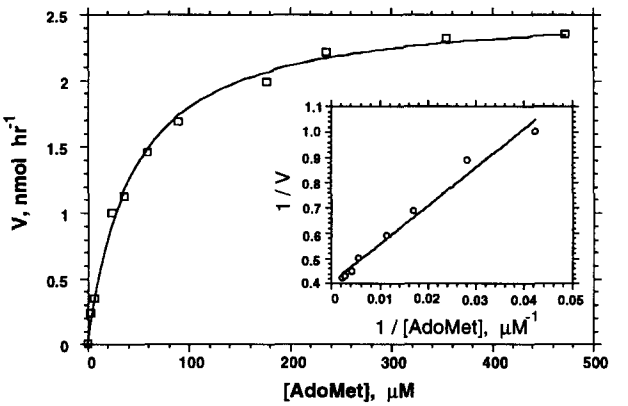

Fig. 3. Determination of kinetic constant of a double deletion mutant (NCdel-1). ACC synthase activity was measured at different substrate concentrations as indicated. Each point represents an average value of five replicates determined using the highly purified enzyme. The hyperbolic curve of the enzyme activity was drawn by ENZFITTER software based on the Michealis-Menten equation, $v = V_{max} \times [S]/(K_m + [S])$. The double-reciprocal plot (inset) of NCdel-1 was drawn by the same software based on the Lineweaver-Burk equations, $1/v = (K_m/V_{max}) \times (1/[S]) + 1/V_{max}$.

Table 1
Oligonucleotides used to synthesize *wt* ACC synthase and its deletion mutants

Primers	Oligonucleotide sequence
P_N	AAACC ATG GGA TTT GAG ATT GCA AAG ACC
N1	TATACC ATG GCT TTA TCA AAA TTG GCT ACT AAT G
N2	TATACC ATG GCT TCA AAA TTG GCT ACT AAT GAA GAG
N3	TATACC ATG GCT AAA TTG GCT ACT AAT GAA GAG CA
N4	TATACC ATG GCT TTG GCT ACT AAT GAA GAG CAT
N5	TATACC ATG GCT AAC TCG CCA TAT TTT GAT GGG
N6	TATACC ATG GGA TAT TTT GAT GGG TGG AAA GCA TAC
N7	TATACC ATG GGA TTT GAT GGG TGG AAA GCA TAC GAT
N8	TATACC ATG GAT GGG TGG AAA GCA TAC GAT AG
C1	AAG GAT CCT TAA GTT TTC TCA ACA CCT ACG AAC C
P_c	TATA GGA TCC TTA ACG AAC TAA TGG TGA GGG AGG

The underlined sequences are the genetically engineered restriction sites *Nco*I or *Bam*HI used to facilitate cloning of PCR product into pET11 d vector. P_N and P_C primers were employed to direct the synthesis of cDNA encoding *wt* ACC synthase in PCR.

the enzyme extract; second, the mutant enzymes without Ser-13 are enzymatically less active but are capable of producing comparable amount of ACC during a longer period of culture time. It is possible that the amount of the substrate AdoMet available to ACC synthase becomes limited after the addition of IPTG and rifampicin. Thus, a continuing increase in the level of expressed target protein may not lead to a proportional increase in ACC accumulation. In agreement with ACC content assay, further deletion through Tyr-27 resulted in the abolition of the enzyme activity.

In the C-terminal deleted mutants, it was reported that such deletions led to an increased enzyme activity and K_m [10]. It is possible that this may also be true in Ndel-1, -2 and NCdel-1, -2 mutants in which an amino acid region (aa-2 to -12) was deleted. To gain more information, we selected the ACC synthase mutant, NCdel-1, for further analysis because Cdel was reported to have a high K_m value of 280 μM. Hence, if deletion of an amino acid region from the N-terminus changes the substrate affinity property of the resulting enzyme, it can be detected easily. NCdel-1 was purified 30-fold via 35–70% ammonium sulfate differential precipitation, DEAE and Superose 12 columns on fast protein liquid chromatography. Using this ACC synthase preparation with a specific activity of 34,000 units per mg of protein, the K_m was determined to be 42 μM (Fig. 3). In comparison to the previously published K_m of 280 μM for Cdel and that of 22 μM for *wt* ACC synthase [10], it is closer to that of wild type than to that of Cdel. These data indicated that the double deletion mutant, NCdel-1, is of higher substrate binding affinity than that of Cdel. Taken together, these results demonstrated that both the non-conserved N-terminal and C-terminal region affect ACC synthase function. Since it was reported previously that deletion of an amino acid region (aa-434 to -485) from the C-terminus of ACC synthase resulted in a monomeric enzyme of enhanced substrate affinity [10], it may be speculated that double deletion of two amino acid regions (aa-2 to -12 and aa-434 to -485) from both the N-terminus and the C-terminus of wound-induced tomato fruit ACC synthase (LE-ACS2), respectively, might generate a more efficient enzyme.

The activity of ACC synthase can be induced 10-fold by wounding and treatment with LiCl [8]. This increase in activity is due to de novo synthesis of this enzyme [20]. It was, therefore, suggested that ACC synthase is transcriptionally regulated. Northern blot analysis of ACC synthase transcripts in wound-induced tissue indicated that it becomes detectable after 20 min of wounding and peaks around 8 h of wounding [21]. In one case, the transcripts appear in a transient fashion and peak in 30 min and disappear by 3 h [22]. Whether ACC synthase activity is also regulated at post-translational level is not clear. There is no direct evidence either for or against the post-translational control of ACC synthase activity. In one recent report, ACC synthase activity was shown to be affected by the kinase- and phosphotase-inhibitors [23]. It is noteworthy that in zucchini, the in vitro translation of mRNA and in vivo labeling of ACC synthase followed by immunoprecipitation showed a similar size of 53 kDa subunit as determined by SDS-PAGE [11]. However, in tomato, in vivo labeling of ACC synthase and in vitro translation of mRNA showed two polypeptides of different size, 47 vs. 56 kDa [15]. Since the size of ACC synthase isolated directly from plant tissue is smaller than that of the predicted full-length ACC synthase, the data are interpreted as either that a limited processing of newly synthesized ACC synthase happens in vivo or that the limited proteolysis occurs during enzyme isolation procedure. Clearly, more experiments are needed to elucidate this issue.

Study of the roles of the N-terminus and the C-terminus in ACC synthase function could provide useful information not only for the elucidation of the structure and function relationship of ACC synthase but also in clarifying the mechanism(s) of the regulation of ACC synthase gene expression in plant tissue.

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