Phosphorylation of Tomato 1-Aminocyclopropane-1-carboxylic Acid Synthase, LE-ACS2, at the C-terminal Region*

Received for publication, February 19, 2001, and in revised form, April 18, 2001
Published, JBC Papers in Press, May 24, 2001, DOI 10.1074/jbc.M101543200

Mihoko Tatsuki and Hitoshi Mori†
From the Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho Chikusa-ku, Nagoya, Aichi 464-8601, Japan

1-Aminocyclopropane-1-carboxylic acid synthase is a key enzyme in the ethylene biosynthesis pathway. Recent studies raise the possibility that 1-aminocyclopropane-1-carboxylic acid synthase (ACS) is regulated not only transcriptionally but also post-translationally. To elucidate post-translational ACS regulation, we analyzed the modification of LE-ACS2 protein, a wound-inducible isozyme in the ACS family, in tomato fruit (Lycopersicon esculentum L.) using an anti-LE-ACS2 antibody. We detected phosphorylated LE-ACS2 at 55-kDa using immunoprecipitation from an extract of wounded fruit fed with [32P]Pi. Analysis of LE-ACS2 phosphoamino acids indicated that serine residue(s) were phosphorylated. In vitro phosphorylation analyses using site-directed mutagenesis of recombinant LE-ACS2 as a substrate demonstrate that serine 460 located at the C-terminal region of ACS is phosphorylated. During tomato ripening stages, expression of both LE-ACS2 and LE-ACS4 mRNA increased. LE-ACS4, however, was not phosphorylated in vivo. These results suggest that ACS isozymes have different post-translational regulatory mechanisms, such as phosphorylation.

Ethylene influences numerous plant growth and developmental processes (1). Ethylene is biosynthesized from S-adenosyl-l-methionine (AdoMet) via 1-aminocyclopropane-1-carboxylic acid (ACC). The rate-limiting step in ethylene biosynthesis is the conversion of AdoMet to ACC (2). This step is catalyzed by ACC synthase (ACS; EC 4.4.1.14), which is encoded by a multigene family (3). The ACS genes have been cloned from various species, and their expression patterns have been studied. Distinct subsets of ACS genes are expressed in response to various developmental, environmental, and hormonal factors (3, 4).

Increases in ACS activity under various conditions correlate with increased levels of ACS mRNA, as shown by Northern blot or RNA protection assays, suggesting that ACS is transcriptionally regulated (3). Some reports, however, suggest the possibility that ACS is also regulated post-transcriptionally or post-translationally. Unspliced and spliced LE-ACS3 accumulate in flooded tomato roots (5). In cell suspension cultures, cycloheximide suppresses the increase of ACS activity induced by stress, whereas cordycepin and actinomycin D do not (6, 7).

In other reports, the accumulation of ACS transcripts did not correlate with ACS activity or the rate of ethylene production. This phenomenon was observed in tomato suspension cells treated with elicitor (8), in etiolated seedlings of Arabidopsis mutants eto1 and eto3, which produce elevated levels of ethylene (9), and in cytokinin-treated Arabidopsis etiolated seedlings (10). Although the mechanisms underlying post-transcriptional regulation of ACS genes are still unknown, possible mechanisms of the post-translational regulation of ACS proteins include modifications of ACS that result in a change in activity or stability and mechanism-based inactivation of ACS by the substrate AdoMet (11).

Based on previous studies (12, 13), one possible modification of ACS is a truncation of the C-terminal region, which increases enzyme activity. In previous studies, the molecular masses of some purified ACS isozymes were smaller than those of the in vitro translation products (14–16) and smaller than those of ACSs expressed in Escherichia coli harboring ACS cDNA (17, 18). Therefore, proteolysis of ACS was thought to occur during extraction and purification from plants. Deletion of 52 amino acid residues from the C terminus of ACS increased enzymatic activity (12), whereas deletion of the first 27 amino acid residues from the N terminus of ACS completely abolished ACS activity (13). Based on these results, ACSs are thought to be cleaved at the C-terminal region and proteolytic processing of this region to increase enzymatic activity. There is no experimental evidence, however, that this regulation occurs in vivo.

ACS might also be modified by protein phosphorylation. In tomato suspension cells, a protein kinase inhibitor, K-252a, blocked elicitor-induced ACS activity, and a protein phosphatase inhibitor, calyculin A, induced a rapid increase in ACS activity in the absence of elicitors and accelerated the rate of increase of elicitor-inducible ACS activity (19). An attempt to detect phosphorylated ACS by immunoprecipitation, however, was unsuccessful. Thus, Spanu et al. (19) suggested that protein phosphorylation/dephosphorylation was involved in the regulation of ACS not by regulating the catalytic activity but by controlling the rate of enzyme turnover. Tuomainen et al. (20) observed the same phenomenon in ozone-exposed tomato leaves and suggested that protein phosphorylation/dephosphorylation regulated ACS activity. These results were obtained using only inhibitors. Therefore, it is still unknown whether ACS was directly phosphorylated or whether other proteins that control ACS activity or stability were phosphorylated.

To understand post-translational ACS regulation, we used the wound-inducible isozyme in tomato, LE-ACS2, and an anti-LE-ACS2 antibody. Crude extracts from wounded tomato fruit fed with [32P]Pi were immunoprecipitated. We report here that LE-ACS2 is phosphorylated at Ser-460 at the C-terminal re-
Phosphorylation of ACC Synthase

Plant Material—Tomato fruit (Lycopersicon esculentum L. cv. Super First) was obtained from a local market or harvested at the Aichi Agricultural Research Center. Wounded tissues were prepared by slicing pericarp tissues (1.0 × 0.3 × 0.3 cm³) and incubating them for 3 h at 25 °C.

Recombinant of Wild-type and Mutant LE-ACSs—The following sets of primers were used for polymerase chain reaction to amplify DNA fragments of LE-ACS2, -3, and -4.

**Forward primers:**
- LE-ACS2: 5’-CGCGGATCCATGGGATTTGAGATTG-3’
- LE-ACS3: 5’-TTTCTCGAGGCTAAGACAAATTATGATGTC-3’
- LE-ACS4: 5’-GGGATCCATGGATTTGGAGACGAGT-3’

**Reverse primers:**
- LE-ACS2: 5’-CGCGGATCCATGGGATTTGAGATTG-3’
- LE-ACS3: 5’-TGCTTCTCCCAAATCTTGAGAAGACTTGGTTTTTCGAAAAG-3’
- LE-ACS4: 5’-GAATAATTTGAGACTTGGTTTTTCGAAAAGTTATTC-3’

For production of the anti-LE-ACS2 antibody, the C-terminal region of LE-ACS2 was affinity-purified with TALON™ metal affinity resin (CLONTECH). The phosphorylated protein band was excised from the polyvinylidene difluoride membrane (Hybond-P, Amersham Pharmacia Biotech). The phosphorylated protein band was excised from the polyvinylidene difluoride membrane and partially hydrolyzed in 6 N HCl for 1 h at 110 °C (27). The hydrolyzed amino acids were separated using two-dimensional thin-layer electrophoresis on a P80 cellulose plate (Sigma, St. Louis, MO, USA). The two-dimensional thin-layer plates were sprayed with ninhydrin (Ninhydrin spray; Wako, Tokyo, Japan) to locate the phosphoamino acid standards and then placed on an imaging plate for 1 week to detect the radiographic image as described above.

In Vitro Phosphorylation—Incorporation of [γ-32P]ATP into SA-204 protein was performed in a kinase reaction mixture (40 μl) that consisted of 70 pmol of purified recombinant LE-ACS2s as substrate proteins, 1 μg of the kinase fraction, and 0.1 mM [γ-32P]ATP (0.22 nCi/μmol) in Buffer D with 0.1 mM CaCl₂ or 0.5 mM EGTA. The reaction was incubated at 30 °C and stopped by adding SDS sample buffer. The polypeptides were separated using SDS-PAGE. Radioactivity incorporated in the LE-ACS2 isoforms was detected as described above.
remained enzymatically active.

suggest that the truncated form, the 49- or 47-kDa polypeptide, of the extracts prepared with proteinase inhibitors was detected (Fig. 1A, left) as in A, lane 1). Molecular mass markers are indicated at the left of the lane.

Dephosphorylation by PP2A and λ-PPase—The 30 to 75% (NH₄)₂SO₄ fractions of the wounded fruit extracts were dissolved in Buffer F (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 μM PLP, Complete™) and desalted on PD 10 columns that were pre-equilibrated with Buffer F. This sample was incubated in PP2A reaction mixture (2 mM MOPS-KOH, pH 7.5, 15 mM NaCl, 6 mM β-mercaptoethanol, 0.1 mM MgCl₂, 0.1 mM EGTA, 0.01 mM MnCl₂, 1% glycerol, 0.01 mg/ml bovine serum albumin, 10 μM PLP, Complete™, 0.04 units of purified PP2A from human red blood cells (Upstate Biotechnology)) or in λ-PPase reaction mixture (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol, 0.01% Brij 35, 2 mM MnCl₂, 10 μM PLP, Complete™, 80 units of recombinant λ-PPase (New England BioLabs)) for 10 min at 30°C. The protein samples (100 μl) were added to 500 μl of ACS assay buffer (25), and the activity was measured.

RESULTS

We prepared an anti-LE-ACS2 antibody using recombinant LE-ACS2 expressed in E. coli as the antigen. To confirm that the purified antibody recognized LE-ACS2, Western blot analysis was performed with wounded fruit extract. Wounded pink tissues were homogenized with proteinase inhibitors to prevent proteolysis. The anti-LE-ACS2 antibody specifically recognized a 55-kDa polypeptide (Fig. 1A, left). Next, the ACS activity was assayed in wounded fruit extracts before and after immunoprecipitation. The anti-LE-ACS2 antibody completely precipitated the wound-induced enzymatic activity, whereas the preimmune IgG did not (data not shown). This result indicated that the anti-LE-ACS2 antibody was able to immunoprecipitate the native form of LE-ACS2 in the fruit extracts.

Previous reports suggested that proteolytic processing of ACS occurs in vivo and in vitro (3). Our finding that LE-ACS2 was a 55-kDa polypeptide (Fig. 1A, left), the molecular mass that corresponds to the one calculated from the deduced amino acid sequence, suggested that proteolytic processing did not occur in vivo. To clarify how the apparent proteolytic processing occurred, we prepared wounded fruit extracts without proteinase inhibitors and analyzed them using Western blot analyses. In this sample, 49- and 47-kDa polypeptides were detected (Fig. 1A, right). ACS activity was detected in both extracts, which were prepared with or without proteinase inhibitors; the activity (+ standard deviation; units/mg total protein) of the extracts prepared with proteinase inhibitors was 7.59 ± 0.31 and that without was 10.6 ± 0.37. These results suggest that the truncated form, the 49- or 47-kDa polypeptide, remained enzymatically active.

To further clarify whether the 49- or 47-kDa polypeptides were derived from the 55-kDa polypeptide, a crude extract was prepared with proteinase inhibitors, and the inhibitors were then removed from the extract by gel filtration. When this sample was incubated at 25°C, the amount of the 55-kDa polypeptide decreased with time, and that of the 49- and 47-kDa polypeptides simultaneously increased (Fig. 1B). Thus, the in vivo molecular mass of LE-ACS2 is 55 kDa, and it is converted to the 49- and 47-kDa polypeptides during extraction by proteinases in the absence of proteinase inhibitors. In addition, after the proteinase inhibitors were removed from the extract, LE-ACS2 was readily digested by the trace amounts of proteinases that remained active in the extract, suggesting that LE-ACS2 was easily attacked by proteinases.

To determine whether ACS is phosphorylated in vivo, pericarp tissues of pink fruit were sliced, fed with [32P]Pi, and homogenized with proteinase inhibitors. A 55-kDa [32P]-labeled...
The 32P-labeled phosphoprotein was immunoprecipitated with the anti-LE-ACS2 antibody (Fig. 2A, lane 3) but not with preimmune IgG (Fig. 2A, lane 2). The size of the 32P-labeled phosphoprotein was consistent with the 55-kDa polypeptide that was detected in the total extract using Western blot analysis with the anti-LE-ACS2 antibody (Fig. 2B). Non-radiolabeled recombinant LE-ACS2 expressed in E. coli competed with the 32P-labeled protein in immunoprecipitation (Fig. 2A, lane 4). These results confirm that the 32P-labeled 55-kDa protein is LE-ACS2. When the extraction was performed without proteinase inhibitors, radiolabeled polypeptides were not immunoprecipitated with the anti-LE-ACS2 (data not shown), although the 49- and 47-kDa polypeptides were detectable by the anti-LE-ACS2 antibody in the total extract with Western blot analysis (data not shown). This finding suggests that the smaller proteolytic polypeptides derived from LE-ACS2 were not phosphorylated.

To determine which amino acid residue(s) were phosphorylated, 32P-labeled LE-ACS2 was acid-hydrolyzed, and the resulting amino acids were separated using two-dimensional thin-layer electrophoresis. A radioactive spot was located at the phosphoserine site (Fig. 3), indicating that LE-ACS2 was phosphorylated on serine residue(s).

To establish an in vitro ACS phosphorylation system, purified recombinant LE-ACS2 was used as a protein kinase substrate that was extracted from wounded tomato fruit. The 5 to 20% polyethylene glycol fractions were used as the crude protein kinase fraction, because its specific activity of 335 (nmol of phosphate/min/mg of substrate/mg of protein) was higher than that of the 30 to 60% (NH₄)₂SO₄ fractions of 15 (nmol of phosphate/min/mg of substrate/mg of protein). The molecular mass of recombinant LE-ACS2 is 72 kDa, because tag proteins are also included. Fig. 4 shows the results of in vitro phosphorylation of recombinant LE-ACS2. Incubation of the substrates with ATP and a fraction of the protein kinase resulted in the incorporation of 32P from γ-labeled ATP into the 72-kDa polypeptides in a time-dependent manner (Fig. 4, A and B).
25% of the total recombinant LE-ACS2 was phosphorylated in the *in vitro* ACS phosphorylation system after a 10-min incubation, assuming that the LE-ACS2 possessed only one phosphorylation site. Phosphorylation did not occur in the presence of EGTA (Fig. 4, C and D), indicating that phosphorylation of recombinant LE-ACS2 is Ca\textsuperscript{2+}-dependent. The *in vitro* phosphorylation system was used to determine the phosphorylation site of LE-ACS2 using various mutant LE-ACS2s and synthetic polypeptides as substrates. The C-terminal-deleted LE-ACS2, which was digested by the proteinase during extraction, was not phosphorylated (data not shown), and the deletion mutant LE-ACS2, which lacked 46 amino acids from the C-terminal, was not phosphorylated (Fig. 5B, lane 2), suggesting that the C-terminal region includes the phosphorylation site. To determine which serine residue(s) were phosphorylated, we used various synthetic peptides derived from the C-terminal region of LE-ACS2. Synthetic peptides derived from the C-terminal region included the phosphorylation site. To determine whether other isozymes that have the amino acid sequence surrounding the LE-ACS2 phosphorylation site was conserved in some various ACSs is not conserved, the amino acid sequence near the LE-ACS2 phosphorylation site was conserved in some various ACSs.

Although it has been reported that the C-terminal region of various ACSs is not conserved, the amino acid sequence near the LE-ACS2 phosphorylation site was conserved in some various ACSs (Fig. 8). To determine whether other isoforms that have a phosphorylation site similar to that of LE-ACS2 are phosphorylated, *in vitro* phosphorylation was performed using other tomato isoforms, LE-ACS3 and LE-ACS4. LE-ACS3 has the same sequence as LE-ACS2, and LE-ACS4 does not. Although recombinant LE-ACS3 was phosphorylated similarly to LE-ACS2, LE-ACS4 was not (Fig. 9). These results suggest that LE-ACS3 is also phosphorylated *in vivo* and that isoforms that possess the amino acid sequence surrounding the LE-ACS2 phosphorylation site of the C-terminal region can be phosphorylated.

To determine the effect of protein phosphorylation and dephosphorylation on enzymatic activity, the activity of wound-inducible ACSs, which include mainly phosphorylated LE-ACS2, was assayed after dephosphorylation by PP2A and λ-PPase. The activity (± standard deviation; units/mg of total protein) of the ACS that was treated with PP2A was 4.99 ± 0.42, and that of native ACS was 5.16 ± 0.13. The activity of the ACS that was treated with λ-PPase was 4.01 ± 0.18, and that of native ACS was 4.32 ± 0.12. There was no significant difference between native and dephosphorylated ACS activity.

Furthermore, the specific activity of *in vitro* phosphorylated recombinant LE-ACS2 was the same as that of the non-phosphorylated recombinant LE-ACS2; the activities (± standard deviation) of phosphorylated recombinant LE-ACS2 and non-phosphorylated LE-ACS2 were 153109 ± 2087 units/mg of total protein, respectively. Recombinant LE-ACS2, which was site-directed mutagenized (Ser-460 to Glu) to introduce a negative charge despite phosphorylation, also did not change the specific activity (151328 ± 2087 units/mg of total protein). These results indicate that phosphorylation of LE-ACS2 did not affect enzymatic activity.

**DISCUSSION**

The findings of the present study indicate that LE-ACS2 is phosphorylated on Ser-460 at the C-terminal region. Previous studies using protein kinase and phosphatase inhibitors indicated that protein phosphorylation was involved in the regulation of ACS (19, 20). There was no direct evidence of this, however, at the molecular level. Our results clearly show that protein phosphorylation of ACS occurs *in vivo*. 

**FIG. 6. In vitro phosphorylation of synthetic peptides derived from the C-terminal region of LE-ACS2. A**, synthetic peptides of C-terminal region. **B**, incorporation of radioactivity into synthetic peptides. The peptides were incubated with a protein kinase fraction as described in the legend for Fig. 4 with 0.1 mM Ca\textsuperscript{2+} (black boxes) or 0.5 mM EGTA (white boxes). Error bars denote the standard deviation of the measurement of three independently processed samples.

**FIG. 7. In vitro phosphorylation of LE-ACS2 mutants.** Recombinant LE-ACS2 was incubated with a kinase fraction as described in the legend for Fig. 4. Substrates were wild-type (lane 1), mutant S460G (lane 2), S462G (lane 3), S460, 462G (lane 4), and S460E (lane 5). A, CBB staining; B, radiographic image.
The (F/L)RLS(F/F) sequence is also phosphorylated in other ACSs. Site-directed mutagenesis in ACS5 indicates the deduced substitution of amino acids that caused a single base pair insertion. The accession numbers are as follows: CM-ACS1, D01032; LE-ACS2, M34289; LE-ACS3, U17972; LE-ACS7, U20392; Ps-ACS1, AF016458; Ps-ACS2, AF016459; VR-ACS1, M80554; VR-ACS2, M80555; VR-ACS6, AB000679; AT-ACS2, M95594; AT-ACS4, L29260; and LE-ACS4, M63490.

ACSs are thought to cleave at the C-terminal region, and cleavage of this region increases enzymatic activity (12). The present results indicate that the molecular mass of LE-ACS2 is 55 kDa, however, which is consistent with that calculated from the deduced amino acid sequence (Fig. 1), indicating that cleavage does not occur in vivo. The 55-kDa polypeptide was easily cleaved during extraction with a proteinase, resulting in the 47-kDa polypeptide (Fig. 1). Because both the 47-kDa polypeptide (Fig. 1) and the recombinant ACSs that were truncated at the C-terminal region (12) had enzymatic activity, the 47-kDa polypeptide is probably truncated at the C terminus and not at the N terminus. This is supported by the finding that crystalized apple ACS lacks a segment of ~5–kDa at the C terminus (29).

Although proteolytic processing of the C-terminal region does not occur in vivo, ACS is phosphorylated at the C-terminal region, which was thought to be cleaved from ACS. Our results led us to speculate that the failure of Spanu et al. (19) to detect phosphorylated ACS was because of the deletion of the phosphorylation site from the C terminus during extraction in the absence of suitable or effective proteinase inhibitors. The deduced amino acid sequences of various ACS isozymes reported to contain a single-base pair insertion. The accession numbers are as follows: CM-ACS1, D01032; LE-ACS2, M34289; LE-ACS3, U17972; LE-ACS7, U20392; Ps-ACS1, AF016458; Ps-ACS2, AF016459; VR-ACS1, M80554; VR-ACS2, M80555; VR-ACS6, AB000679; AT-ACS2, M95594; AT-ACS4, L29260; and LE-ACS4, M63490.

The phosphorylation of ACS isozymes. Box 7 is the 7th conserved domain of ACSs. The shadowed box indicates the conserved amino acid sequence that was considered to be the phosphorylation site (asterisk). The underlining indicates the deduced substitution of amino acids that caused a single base pair insertion. The accession numbers are as follows: CM-ACS1, D01032; LE-ACS2, M34289; LE-ACS3, U17972; LE-ACS7, U20392; Ps-ACS1, AF016458; Ps-ACS2, AF016459; VR-ACS1, M80554; VR-ACS2, M80555; VR-ACS6, AB000679; AT-ACS2, M95594; AT-ACS4, L29260; and LE-ACS4, M63490.

ACS phosphorylation site. Characterization of the specific protein kinases involved and the determination of substrate specificity are necessary to further understand the phosphorylation of ACSs.

Spanu et al. (19) reported that although treatment with protein kinase and phosphatase inhibitors affected ACS activity, the ACS activity in the extracts from elicitor-treated cells was not affected by protein phosphatases PP1 and PP2A from rabbit muscle or alkaline phosphatase (19). In the present study, treatment of extracts from wounded tomato fruit with PP2A and λ-PPase did not affect ACS activity. Furthermore, the enzymatic activity of recombinant LE-ACS2 did not change after in vitro phosphorylation. Thus, we suggest that phosphorylation of ACS does not change enzymatic activity.

The possibility that phosphorylation of ACS regulates ethylene production is supported by the finding that alteration of the C-terminal region of Arabidopsis ACS5 induces the eto2–1 mutant to overproduce ethylene (10). Vogel et al. (10) reported that eto2–1 has a single base pair insertion 35 base pairs upstream from the stop codon and that this frameshift mutation was predicted to change the 12 terminal amino acids of ACS5 from ERSSERLELSLKERTVSNWY to FGRDRRSSCT. Comparing the deduced amino acid sequence among the various ACS isozymes, ACS5 could be phosphorylated at RVG, that is, Ser-400, in vivo. If this is the case, the eto2–1 version of ACS5 lost the phosphorylation site. Because alteration of this region resulted in a 20-fold increase in ethylene production in eto2–1 relative to the wild-type, Vogel et al. (10) suggested that this domain acts as a negative regulator of ACS5 function. Although mutation of the C-terminal region of ACS5 leads to increased ethylene production, the eto2–1 version of ACS5 had a specific activity comparable with that of the wild-type expressed in E. coli. It seems that the role of ACS phosphorylation is not to regulate specific activity. Spanu et al. (19) reported that a protein kinase inhibitor blocked elicitor-induced ACS activity, and a protein phosphatase inhibitor increased ACS activity. Furthermore, Ecker's group recently identified the ETO1 gene (31). They reported that the ETO1 protein bound to the C-terminal region of ACS5 and inhibited its activity. These reports and our results suggest that the C-terminal domain has a pivotal role in the regulation of ACS turnover. One possible mechanism of regulation by phosphorylation is that the non-phosphorylated C-terminal region of ACS might be the target of some proteins (e.g., ETO1) involved in a degradation system, such as the ubiquitin system. The loss of the deduced phosphorylation site, such as the eto2–1 version of ACS5, might prevent the binding of some proteins, whereas phosphorylation might impede the binding of some proteins to the C-terminal domain. Consequently, phosphorylated ACS would be protected from the degradation system, the turnover of phosphorylated ACS would be prolonged, ACS would accumulate, and ACS activity would increase, especially in the case of the explosive increase of ACS activity following stimulation. The change of the C-terminal sequence (e.g., eto2–1) might
mimic this phenomenon. Further study of the difference in turnover on non-phosphorylated and phosphorylated ACS and the identification of protein kinase(s), protein phosphatase(s) involved in ACSs phosphorylation states, and hypothetical binding proteins are needed.

Because the sequence surrounding the phosphorylation site is conserved in various ACSs, phosphorylation might be a general feature of the ACSs in other species besides tomato. Although we could not determine the physiologic role of LE-ACS2 phosphorylation, the present results suggest that different isozymes are regulated by different phosphorylation states. Of particular interest is that LE-ACS4 does not have the deduced phosphorylation site (Fig. 8) and is not phosphorylated in vitro (Fig. 9). During tomato-ripening stages, expression of both LE-ACS2 and LE-ACS4 mRNAs increase. Although the contribution of LE-ACS2 and LE-ACS4 enzyme activities to the production of ethylene during ripening stages has not been demonstrated, these isozymes might have different post-translational regulatory mechanisms, such as phosphorylation. To clarify how ACS is regulated by its phosphorylation state, further studies are needed to determine whether the phosphorylation of ACS is reversible and whether other ACS isozymes are phosphorylated.

In conclusion, the tomato wound-inducible ACS isozyme, LE-ACS2, is phosphorylated at the C-terminal region, which was previously thought to be truncated. The present study will help to elucidate the regulatory mechanism of ethylene biosynthesis by post-translational regulation of ACS.

Acknowledgments—We thank Dr. S. Huber and Dr. K. Inui for valuable discussions. We also thank S. Sugawara, from the Aichi Agricultural Research Center, and Dr. M. Nagata, from the National Research Institute of Vegetables, Ornamental Plants, and Tea, for providing tomato samples.

REFERENCES

1. Abeles, F. B., Morgan, P. W., and Saltveit, M. E., Jr. (1992) Ethylene in Plant Biology, Academic Press, San Diego, CA

2. Adams, D. O., and Yang, S. F. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 170–174

3. Kende, H. (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 283–307

4. Zarembinski, T. I., and Theologis, A. (1994) Plant Mol. Biol. 26, 1579–1597

5. Olsen, D. C., Oetiker, J. H., and Yang, S. F. (1995) J. Biol. Chem. 270, 14056–14061

6. Chappell, J., Halibrock, K., and Boller, T. (1984) Plant Mol. Biol. 161, 475–480

7. Felix, G., Grosskopf, D. G., Regenass, M., Basse, C. W., and Boller, T. (1991) Plant Physiol. 97, 19–25

8. Oetiker, J. H., Olsen, D. C., Shi, O. Y., and Yang, S. F. (1997) Plant Mol. Biol. 34, 275–286

9. Woeste, K. E., Ye, C., and Kieber, J. J. (1999) Plant Physiol. 119, 521–530

10. Vogel, J. P., Woeste, K. E., Theologis, A., and Kieber, J. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 275–280

11. Li, N., and Mattoo, A. K. (1994) J. Biol. Chem. 269, 6908–6917

12. Reed, J. S., and Mattoo, A. K. (1994) J. Biol. Chem. 269, 4859–4863

13. Li, N., Huxtable, S., Yang, S. F., and Kung, S. D. (1996) FEBS Lett. 378, 286–290

14. Nakajima, N., Nakagawa, N., and Imaseki, H. (1988) Plant Cell Physiol. 29, 989–998

15. Sato, T., and Theologis, A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6621–6625

16. Edelman, L., and Kende, H. (1990) FEBS Lett. 282, 635–638

17. Van der Straeten, D., Van Wiezeersch, L., Goodman, H. M., and Van Montagu, M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4859–4863

18. Sato, T., Oeller, P. W., and Theologis, A. (1991) J. Biol. Chem. 266, 3752–3759

19. Sano, P., Grosskopf, D. G., Felix, G., and Boller, T. (1994) Plant Physiol. 106, 529–535

20. Tuomainen, J., Betz, C., Kangasjarvi, J., Ernst, D., Yin, Z.-H., Langebartels, C., and Sandermann, H., Jr. (1997) Plant J. 12, 1115–1126

21. Tatsuki, M., and Mori, H. (1999) Plant Physiol. 120, 709–715

22. Sambrook, J., and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, 3rd Ed., pp. 13.36–13.39, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

23. Ichihara, Y., and Kurosawa, Y. (1993) Gene 130, 153–154

24. Vaitukaitis, J. L. (1981) Methods Enzymol. 73, 46–52

25. Yoshii, H., and Imaseki, H. (1981) FEBS Lett. 140, 270, 275–280

26. Lizada, M. C. C., and Yang, S. F. (1979) Anal. Biochem. 98, 153–154

27. King, M. M., Fitzgerald, T. J., and Carlson, G. M. (1983) J. Biol. Chem. 258, 100, 119, 22–27

28. Kamps, M. D., and Sefton, B. M. (1989) Anal. Biochem. 176, 529–535

29. Kende, H. (1993) Planta 182, 153–154

30. Halford, N. G., and Hooley, R., eds (Shewry, P. R., Halford, N. G., and Hooley, R., eds) pp. 3–15, Oxford University Press, Oxford

31. Cosgrove, D. J., Gilroy, S., Kao, T.-H., Ma, H., and Schultz, J. C. (2000) Plant Physiol. 124, 499–505
