C-terminal phosphorylation is essential for regulation of ethylene synthesizing ACC synthase enzyme

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The genetic and molecular biological studies mainly in Arabidopsis and in some other plants have begun to uncover the various components of ripening signaling pathway in plants. Although transcriptional regulation of major ripening genes have been studied in detail, information on role of phosphorylation in regulating the activity and stability of core ripening pathway associated proteins in relation to ethylene biosynthesis during fruit ripening is still limited. Recently we have demonstrated the evidence for post-translational regulation of MA-ACS1 (*Musa acuminata* ACC synthase 1), the rate limiting step enzyme regulating ripening ethylene production in banana, through phosphorylation at the C-terminal Ser 476 and 479 residues by a 41-kDa Ser/Thr protein kinase.[1] Here we have further discussed role of protein phosphorylation in regulation of stability and activity of ACS enzymes and the mechanistic and evolutionary perspective of phosphorylation pattern of Type I ACC synthase enzymes.

Post-Translation Regulation of ACC Synthase Enzymes through Phosphorylation

Ethylene is a well-known gaseous plant hormone involved in many aspects of plant growth and developmental process including its pivotal role in fruit ripening. ACC synthase (ACS), which converts AdoMet (S-adenosyl-l-methionine) to ACC (1-aminocyclopropane-1-carboxylic acid) acts as the rate limiting step enzyme in ethylene biosynthesis pathway.

ACS genes have been cloned from various plant species. Genome wide analyses have revealed existence of 12 ACS genes in Arabidopsis, 6 in rice, 10 in grapevine and 11 in poplar respectively. Chromosomal distribution studies suggested that ACS genes are dispersed throughout the respective genomes of the four species, while segmental duplication, tandem duplication and transposition events have been shown as the three major factors contributing to this gene family evolution. Whereas segmental depilation was found to play key role in Arabidopsis and poplar, transposition events were more predominant in rice and grapevine.[2] As like tomato, banana ACS is encoded by a small gene family and the members of which are differentially regulated in tissue specific manner during plant development. Although at least nine ACC synthase genes (*MACS1-9*) exist in banana genome, only *ACS1* (*MA-ACS1* (*Musa acuminata* ACC synthase 1) is expressed during banana fruit ripening and has been found to be inducible by exogenous ethylene treatment, suggesting that *MA-ACS1* is a key member of the ACC synthase gene family related to banana fruit ripening.[3] *MA-ACS1* (*Musa acuminata* ACC synthase 1) is encoded by a single gene in banana genome and has been found to be regulated by various abiotic factors like light, cold, wounding and phytohormones including ethylene and auxin at the transcriptional level.[4]

Transcriptional regulation of ACS genes has been studied in different plant species in response to various hormonal, developmental and environmental factors.[3] However, considerable evidences have also

indicated posttranslational regulation of ACS proteins.[5] Previous reports have demonstrated posttranslational regulation of ACS proteins by phosphorylation and targeted protein degradation, controlling the enzyme turnover rate instead of regulation of enzyme catalytic activity.[6] In tomato cell suspension culture, elicitor induced ACS activity has been found to be blocked by a protein kinase inhibitor, while a phosphatase inhibitor caused rapid increase in ACS activity in the absence of elicitors and also enhanced the elicitor inducible activity of ACS.[7] Post-translational regulation of LeACS2 (*Lycopersicon esculentum* ACS2) by phosphorylation in wounded tomato fruit tissue has been reported previously.[8] Biochemical studies indicated that phosphorylation/dephosphorylation of LeACS2 plays key role in regulating the turnover of this protein, upstream of the ubiquitin-26Sproteasome degradation system. Such regulation has been found to be essential in controlling ethylene production in tomato fruit tissue. Furthermore, LeACS2 has been shown to be phosphorylated by CDPK and MAPK at different sites in response to a wounding signal. Phosphorylation at both sites was found to be crucial for LeACS2 stability.[9] In cotton, ACC synthase 2 is phosphorylated by a calcium-dependent protein kinase 1 during cotton fiber elongation,[10] while Arabidopsis MPK3 and MPK6 have been implicated in phosphorylation of Type 1 ACC synthase (ACS2 and ACS6) and in regulation of expression of *AtACS2* and *AtACS6* genes through binding with another transcription factor, WRKY33.[11] Interestingly, in Arabidopsis, genetic and molecular analyses have revealed role of protein phosphatase 2A (PP2A) in positively regulating the abundance of Type 2 ACS proteins, thus providing a sophisticated regulation of overall ethylene production by differentially regulating the turnover and thus stability of specific classes of ACS enzymes.[12] Our recent report has indicated phosphorylation of MA-ACS1, a Type 1 ACS enzyme, at the C-terminal serine 476 and 479 residues by a 41-kDa Ser/Thr family of protein kinase. We found that MA-ACS1 phosphorylation could be associated with the stability of the protein.[1] Together all these findings have indicated posttranslational

modification of ACS enzymes by phosphorylation which seems to play important role in regulating stability of ACSs.

The C-Terminal Region of ACC Synthase is Crucial for Phosphorylation

One of the crucial components in ethylene biosynthesis cascade is the stability of ACS protein, which appears to be correlated positively with ACS protein activity and ethylene production. Previous studies have revealed that the C-terminal region of ACS proteins plays the key role in the regulation of ACS turnover and activity.[8,9] Phytohormones like cytokinins and brassinosteroids have also been found to stabilize multiple ACS proteins and the stabilization has been shown to be C-terminus dependent.[13] Phosphorylation of ACS proteins at the C-terminal region has been found to be essential in regulation of ACS protein stability. Studies with *eto* mutants in Arabidopsis have contributed significantly to our knowledge on mechanism of the posttranslational regulation of ACS. The *eto2* mutation contains a single base-pair insertion which changes the C-terminal 12 amino acid residues in AtACS5, while the *eto3* mutation carries a single amino acid replacement (Val to Asp) at the C-terminal region of AtACS9. Both mutations have shown to confer increased ACS stability in Arabidopsis. Study of *eto1* mutation revealed role of ETO1 (ethylene overproducer 1) in AtACS5 stability in Arabidopsis. ETO1 has been shown to recognize and interact directly with the C-terminal region of AtACS5 and suppresses ACS activity, allowing CUL3 (Cullin 3), a component of ubiquitin E3 ligase, to bind AtACS5, which eventually leads to AtACS5 degradation via the ubiquitin-26S-proteasome complex. The myc-tagged AtACS5 showed increased half-life in *eto1* mutant than wild-type plants.[9] ETO1 and EOL1 (ETO1-like) were found to be specific for Type 2 ACS proteins in Arabidopsis, and the level of ethylene synthesis has been shown to be increased at the maximum level by elimination of ETO1, then EOL2 and finally EOL1.[13] In Rice, 14-3-3 protein was found to interact directly with ACS1 in yeast two-hybrid assays. Rice 14-3-3 proteins

have been suggested to play important role in the regulation of ACS activity via binding to the phosphorylated C-terminal tails of ACSs, therefore protecting them from the degradation during ethylene biosynthesis.[14] In Arabidopsis, hormonal and mutational analyses have shown that the C-terminal region of the Type 2 ACS proteins play key role in regulating the protein stability and thus results in increased ethylene synthesis.[13] These findings have demonstrated that the C-terminal region of ACS is involved in regulating ACS stability.

The C-terminal Ser Residues Constitute the Phosphorylation Sites of ACS Proteins

The deduced amino acid sequences of different isozymes of ACS have indicated that despite high degree of similarity in the rest of protein, the C-terminal regions of ACSs may not be always conserved. The ACS proteins fall into three categories, Type 1, 2 and 3 respectively, specifically based on the amino acid sequence homology of C-terminal region. In addition, the ACS proteins have been shown to possess the (F/L)RLS(F/L) motif at the C-terminal region. Type 1 ACS isozymes contain the Ser residue in the "RLSF" motif, important for CDPK phosphorylation, followed by a long C-terminal tail (27–33 aa) with the three conserved Ser residues which act as the targets of MPK6 phosphorylation.[5] In plum (*Prunus sativus*), predicted proteins of both Ps-ACS4 and Ps-ACS5 are members of Type1 ACS isozymes. However, these predicted proteins (also closely related to Arabidopsis AtACS2 and AtACS6) lack the Val residue in the conserved C-terminal "WVF" motif. On the other hand, predicted protein Ps-ACS1, which belongs to Type 2 ACS isozymes, has been shown to contain a conserved TOE sequence, the "WVF" sequence motif just before the "RLSF" motif and then followed by a short C-terminal tail.[15] Type 2 ACS isozymes, which mainly include the Arabidopsis and tomato ACS proteins, are regulated at the posttranslational level by CDPK phosphorylation.[16] Type 3 isozymes, which include the predicted protein Ps-ACS3, contain a long C-terminal or a short C-terminal tail,
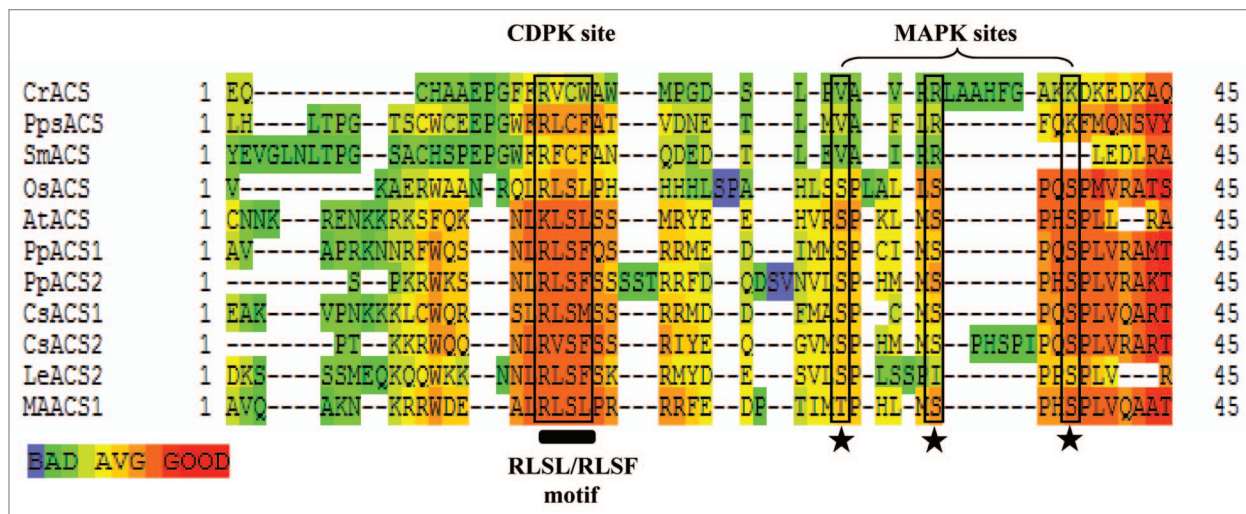
Figure 1. Comparison of C-terminal region of Type I ACC synthase proteins (reported and predicted) from different plant species. *In silico* analyses was performed using by using T-COFFEE server for comparative analysis of the C-terminal sequence of type I ACS proteins from diverse plant genomes. Cr (*Chlamydomonas reinhardtii, v5.3* gene-g7098), Pps (*Physcomitrella patens*, gene-Pp1s235_83V6), Sm (*Selaginella moellendorffii*, gene-75495), Os (*Oryza sativa*, gene-LOC_Os04 g48850), At (*Arabidopsis thaliana*, gene-AT3G61510), Pp1 and Pp2 (*Prunus persica*, gene-ppa016458 min.g and ppa004774 min.g), Cs (*Citrus sinensis*, gene-orange1.1g011570 min.g and 1.1g011801 min.g), Le (*Solanum lycopersicum*, gene-NM_001247249), MA (*Musa acuminata*, gene-AY702076). The putative "RLSF/RLSL" motif (CDPK site, (within the box, indicated by bold black line) and the three Ser residues after the "RLSF/RLSL" motif (MAPK sites indicated by asterisk symbol) have been represented.

while lack all the residues important for phosphorylation.

Evolutionary Perspective of ACS Protein Phosphorylation

In Arabidopsis and tomato, ACS proteins are found to be phosphorylated at specific target residues in the conserved C-terminal region.[8,9,16] Such studies have suggested a similarity in the pattern of phosphorylation of ACS protein either at the CDPK or MAPK or both sites in the C-terminal region of ACSs in higher plants. This may indicate an evolutionary conservation of the phosphorylation target residues at the C-terminal region of ACS proteins as a part of mechanism of the post-translation regulation. A comparative analysis of the partial C-terminal sequence of Type 1 ACS proteins from different plant species have indicated that the CDPK phosphorylation site RLSF/RLSL motif and MAPK phosphorylation sites (the three Ser residues after the "RLSF/RLSL" motif) were comparatively more conserved in higher plant genomes including both monocot and dicot plant species (**Fig. 1**), while such phosphorylation sites were found to be less conserved in lower group of plants. In the unicellular green alga, *Chlamydomonas reinhardtii,* and in bryophytes including

the model species *Physcomitrella patens* and club moss *Selaginella moellendorffii*, the serine residue within the RLSF/RLSL motif has been found to be replaced by cysteine, while the serine residues for putative MAPK sites were replaced by arginine and lysine residues, respectively, suggesting distinct pattern of evolution of CDPK and MAPK phosphorylation sites from lower group of plants to land plants.

Summary

The above discussion provides insight into the possible mechanism of post-translational regulation of ACC synthase, one of the key regulatory proteins in the ethylene mediated ripening signaling cascades. Majority of the studies have provided evidence to argue for the role of phosphorylation/dephosphorylation in regulating the ACS protein stability instead of influencing the catalytic activity. This idea was also supported in our study where accumulation of phosphorylated MA-ACS1 was observed in response to ripening during the onset of climacteric in banana fruit, suggesting that MA-ACS1 seems to be stabilized by phosphorylation to ensure enzyme activity during climacteric phase of ripening. However, further detailed research is required to fully understand the mechanism of phosphorylation of MA-ACS1 in regulation of stability and catalytic activity of this protein in relation to ethylene biosynthesis during banana fruit ripening.

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