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Interaction between ACC Synthase 1 and 14-3-3 Proteins in Rice: a New Insight

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Abstract—In this study, the interaction between rice 14-3-3 protein and 1-aminocyclopropane carboxylic acid synthase (ACS) was observed in yeast cells using yeast two-hybrid assays. Given the fact that 14-3-3 proteins generally bind to their target proteins in a phosphorylation-dependent manner, a hypothesis regarding the regulatory role of 14-3-3 proteins in the activation of ACS is proposed in which 14-3-3 proteins may bind to the phosphorylated C-terminal tails of ACSs and help them to escape from their fated degradation when ethylene biosynthesis is needed. It is reasonable to believe that 14-3-3 protein may play an important role in regulating ACS activity.

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The simple gas ethylene controls various processes in plant growth and development, in addition to its functions in plant responses to both biotic and abiotic stresses [1]. Almost all plant species have the ability to produce ethylene by themselves, and the biosynthetic pathway of ethylene has been studied in detail. It is now clear that ethylene is synthesized from methionine via two intermediates, S-adenosyl-L-methionine (SAM) and 1-aminocyclopropane-1-carboxylate (ACC), and then ACC is oxidized by ACC oxidase to generate ethylene. The conversion of SAM to ACC is catalyzed by ACC synthase (ACS) and this is regarded as a rate-limiting step in the ethylene biosynthesis pathway. Therefore, ACS has been suggested to be an important regulator in the ethylene biosynthesis pathway [2].

It is known that *ACS* genes, as a multiple gene family, are present in most plant species, and the transcription of these genes is differently regulated in different development events and stress conditions [3]. In some cases, ethylene production seems to correlate with increased transcription of *ACS* genes [1]. However, recent studies have indicated that post-transcriptional regulation is another important aspect of the control of ACS activity and ethylene level. In suspension culture cells of parsley and tomato, the increase of ACS activity in response to an elicitor was unaffected when inhibitors of RNA transcription were used, suggesting that a post-transcriptional mechanism existed in the elevation of ACS activity [4]. Molecular genetic studies of *Arabidopsis* ethylene-overproducer (*eto*) mutants further uncovered a regulation mechanism of ACS stability and activity by ETO1 protein. ETO1 was identified in the *eto1* mutant, and research showed that the C-terminal TPR domain of this protein directly interacted with the C-terminus of AtACS5. The N-terminal BTB domain of ETO1 also interacted with AtCUL3, a constituent of E3 ubiquitin ligase complex, by which this protein could serve as a substrate-specific adaptor. The binding of ETO1 resulted in the direct inhibition of AtACS5 activity and/or targeted the enzyme for degradation in a proteasome-dependent manner [5]. Two other *Arabidopsis eto* mutants, *eto2* and *eto3*, were identified to have mutations in the C-terminus of AtACS5 and AtACS9, respectively [6, 7]. Both mutations resulted in the corresponding proteins losing the capability to interact with ETO1, supporting the view that ETO1 plays an important role in the post-transcriptional regulation of ACS protein.

An important question raised by the finding of degradation of ACS through ETO1 interaction is how the activation of ACS proteins is regulated. In regard to this question, one could easily expect that the mechanism for

Abbreviations: ACC) 1-aminocyclopropane-1-carboxylate; ACS) ACC synthase; SAM) S-adenosyl-L-methionine; 3-AT) 3-aminotriazole; CDPK) calcium-dependent protein kinase; MAPK) mitogen-activated protein kinase.

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reversing ACS degradation involves modification of the ACS proteins so that their fate of being targeted to the ubiquitin-26S-proteasome pathway is altered. Actually, protein phosphorylation is the most promising candidate for such a modification. In a previous study, application of inhibitors of protein kinase or phosphatases resulted in a strong effect on the elicitor induction of tomato ACS activity [8]. Recently, a C-terminal serine residue of the tomato ACS isoform LeACS2 was found to be phosphorylated by an unknown calcium-dependent protein kinase (CDPK) in the extracts of wounded tomato fruits [9, 10]. Another study also showed that activation of a stress-response mitogen-activated-protein kinase (MAPK) cascade resulted in the stabilization of AtACS6, and that three serine residues in its C-terminus were phosphorylated by the protein kinase MPK6 [11, 12]. Taken together, these data indicate that plant ACS proteins can be stabilized in response to environmental assaults and developmental signals through phosphorylation by MAPK and/or CDPK cascades.

Although ACS phosphorylation has attracted much interest, the current focus is on the cascades and kinases that perform the modification and the phosphorylated positions in ACC synthase, with the underlying assumption that phosphorylation of ACS protein was sufficient to regulate its activity and/or stability. However, there are a large and growing number of reports indicating that only phosphorylation itself is not enough to cause a change in target protein function. In these cases, the 14-3-3 proteins were required [13]. The common feature of 14-3-3 proteins is that they bind to other proteins in a phosphorylation-dependent manner, and the effects caused by 14-3-3 binding can vary from inhibition to activation of the enzymatic activity of the client, the degradation or protection from degradation of the client, and even the movement of the client from one cellular location to another [14-16]. The 14-3-3 clients are mainly composed of proteins containing phosphoserine or phosphothreonine motifs [13], suggesting that ACS might be one of the 14-3-3 target proteins, and an interaction of ACS and 14-3-3 might occur.

To examine whether the rice 14-3-3 proteins (OsGF14s) could interact with OsACS1, yeast two-hybrid assay was performed using six OsGF14s, and the results showed that the interaction of OsGF14s and OsACS1 could be observed in yeast cells. A hypothesis to explain the specific interaction between ACC synthase and 14-3-3 protein is proposed in this paper. Furthermore, the potential role of 14-3-3 proteins in regulating plant ethylene biosynthesis is discussed.

MATERIALS AND METHODS

Chemicals. RNeasy Plant Mini Kit was purchased from Qiagen GmbH (Germany); RT-PCR system from Promega (USA); *Taq* polymerase and pMD18-T vectors from TaKaRa (China); SD medium and all supplemental materials used in the yeast culture were from BD Biosciences (USA).

Cloning of the full-length cDNA. Six *OsGF14s* and *OsACS1* were cloned from rice tissues according to a method described previously [17]. The following primers were used to clone *OsACS1*: 5′-GATGGTGAGCCAAGTGGTCG-3′ and 5′-GATCATCCGAGACCATCGGG-3′. The GenBank accession numbers of the genes used in this experiment are: *OsGF14b* (U65956), *OsGF14c* (U65957), *OsGF14d* (U65958), *OsGF14e* (AJ276594), *OsGF14f* (AY224434), *OsGF14g* (AK103145), and *OsACS1* (AC135965).

Yeast two-hybrid assays. Yeast strain AH109 was used in this study. Two vectors, pGAD424 and pGBKT7, contained a GAL4 activation domain (AD) and DNA-binding domain (BD), respectively. The entire *OsACS1* was cloned into the pGBKT7 vector after digestion with *Eco*RI and *Bam*HI to generate a pGBKT7[*OsACS1*] construct with a fusion protein of the BD and OsACS1 protein. The autoactivation of ACS1-BD was examined. Then genes of six rice 14-3-3 proteins—*OsGF14b*, *OsGF14c*, *OsGF14d*, *OsGF14e*, *OsGF14f*, and *OsGF14g*—were cloned into the pGAD424 vectors to generate constructs expressing fusion proteins of the AD and different OsGF14s. Additionally, for use as a positive control, pGAD424[*OsACS1*] was also constructed in consideration of the dimeric feature of ACS proteins. Yeast cells were cotransformed with pGAD424[*OsGF14s*] (or pGAD424[*OsACS1*]) and pGBKT7[*OsACS1*] constructs and doubly transformed yeast cells were screened on SD-Leu-Trp solid medium. To determine whether the encoded fusion proteins can interact, the yeast transformants were shaken in liquid SD-Leu-Trp medium until OD_{600} reached 1. Then 5 μl of serial 1 : 10 dilutions were spotted on SD-Leu-Trp and SD-Leu-Trp-His solid medium containing 0, 5, or 10 mM 3-aminotriazole (3-AT). At least three independent experiments were performed, and similar results were obtained.

Sequence analysis and motif searching. The C-terminal amino acid sequences of two OsACSs, nine *Arabidopsis* ACSs, and the HvACS identified in a proteomic study [18] were aligned using the ClustalW program and drawn with GeneDoc. Motif searching was performed by the Scansite program (http://scansite.mit.edu/) and the high stringency setting was selected.

RESULTS AND DISCUSSION

Interaction of rice 14-3-3 isoform and OsACS1 protein. Examining the rice genome revealed eight open reading frames encoding proteins that are highly similar to previously published 14-3-3 proteins in amino acid sequence. We cloned six of them (*OsGF14b-g*) by reverse

transcription and PCR amplification. *OsGF14b-f* belong to the non-ε group, and *OsGF14g* belongs to the ε-like group [17]. Since the 14-3-3 protein generally exerts its power by binding with the target protein, we performed a yeast two-hybrid assay to examine the interaction between OsGF14 and OsACS1. Initially, OsGF14s fused with BD were constructed as the baits in the yeast two-hybrid system. However, the autoactivation of OsGF14-BD compelled us to give up this scheme. This autoactivation could be explained by the interaction of some yeast proteins containing transcriptional activating domain and OsGF14-BD proteins, which was proven by previous reports [19, 20]. Inversely, when OsACS1 was fused with BD, no autoactivation was observed. Then the six *OsGF14s* were fused with *AD* and transformed into the yeast cells carrying *OsACS1-BD* vectors. Additionally, it has been reported that ACS forms a dimeric structure *in vivo* [21]. Therefore, the interaction of OsACS1-AD and OsACS1-BD was utilized as a positive control.

As illustrated in Fig. 1, yeast strains co-transformed with the *OsACS1-AD* and *OsACS1-BD* vectors showed interaction-dependent growth in the SD-Trp-Leu-His medium, indicating the yeast two-hybrid systems functioned well in this experimental system. In the six rice isoforms examined, OsGF14e and OsGF14f interacted with OsACS1 most strongly, followed by OsGF14g, while a slight but considerable interaction of OsGF14b-d and OsACS1 were also observed. Although the difference of expression of OsGF14s could not be excluded, it might be true that 14-3-3 isoform diversity of interaction with OsACS1 did exist. Furthermore, increasing concentrations of 3-AT were added into SD-Trp-Leu-His medium and similar results still could be seen, which confirmed the strength of the OsGF14–ACS interaction. In a recent proteomic study, barley 14-3-3A was expressed in *Escherichia coli* and immobilized on an affinity column to pull down 14-3-3 binding proteins from developing barley grains [18]. The binding was shown to be phosphorylation-dependent by using phosphatase digestion. Intriguingly, the ethylene biosynthetic enzyme ACC synthase was also found in this screening. These interactions demonstrated by yeast two hybridization and pull down experiments might imply some regulation of ethylene biosynthesis through 14-3-3.

Sequence basis for the interaction between 14-3-3 and ACS. It is known that ACS exists as a large protein family in plant species. Based on the length and traits of the C terminus, ACSs are divided into three groups, named type 1, 2, and 3 (Fig. 2). Unlike type 3, both type 1 and type 2 contain the RXSX domain. Compared with type 2 ACC synthases, type 1 ACSs possess longer C-terminal tails after the RXSX motif [10]. Considering the complexity of the plant ACS family, one may question whether the interaction of ACS and 14-3-3s might be restricted to a certain ACS protein or a specific type. Interestingly, the ACS capable of binding to 14-3-3 from barley grain [18] may be type 1, while OsACS1 belongs to type 2 (Fig. 2). Therefore, it is reasonable to believe that the interaction with 14-3-3 might be a general feature of the ACC synthases, at least for type 1 and 2 isoforms.

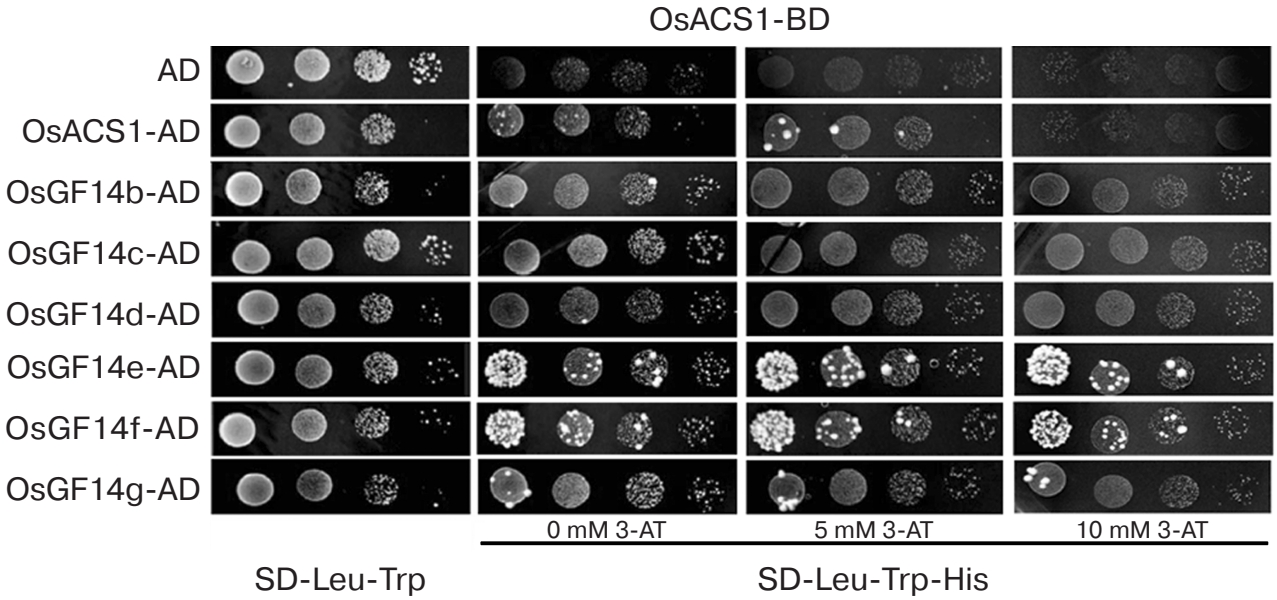

Fig. 1. Interaction of OsGF14s and OsACS1 in yeast two-hybrid assays. *OsACS1* was inserted into pGBKT7 carrying *TRP1* marker to generate OsACS1-BD bait vectors. Six rice 14-3-3 ORFs or *OsACS1* were cloned into pGAD424 with *LEU2* marker to generate OsGF14-AD or OsACS1-AD vectors. OsACS1-BD and various AD fusion vectors were cotransformed into yeast AH109 cells, respectively. The transformants were spotted on SD-Leu-Trp (control) and SD-Trp-Leu-His solid medium containing 0, 5, or 10 mM 3-AT. The cells harboring OsACS1-AD and OsACS1-BD were used as positive control and blank pGAD424 (AD) as negative control.

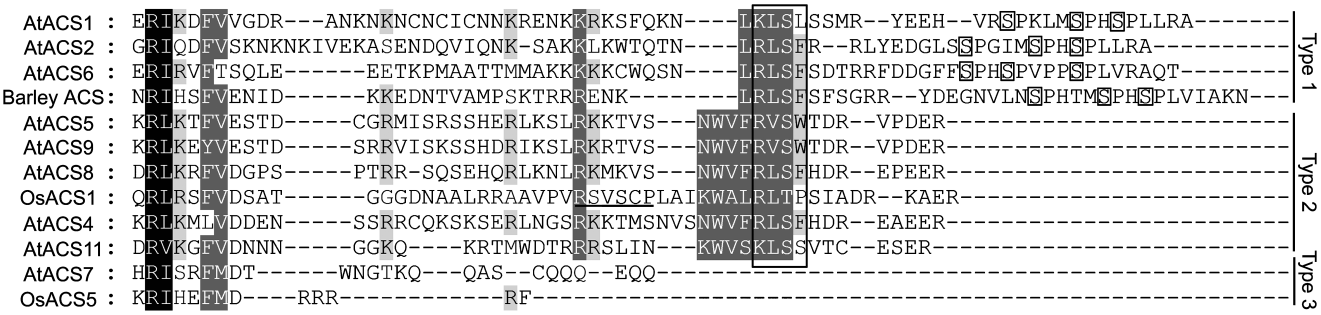

Fig. 2. Alignment analysis of ACC synthases from various plant species. ACC synthases are grouped into three types based on the length and traits of the C terminus. Identical amino acids and conservative changes are indicated by reversed and shaded characters. The RXSX motifs targeted by unidentified CDPK and the serine residues targeted by MPK6 are framed. The conserved 14-3-3-binding motif I in OsACS1 is underlined. The GenBank accession numbers of these proteins are: OsACS1 (AAT77071), OsACS5 (BAB12704), HvACS (P23599), AtACS1 (AAM91649), AtACS2 (AAG50097), AtACS4 (AAC32428), AtACS5 (CAA16680), AtACS6 (AAK27237), AtACS7 (AAG48754), AtACS8 (AAG50090), AtACS9 (AAG48755), and AtACS11 (AAG48768).

In most cases, the regulatory roles of 14-3-3 proteins are played by binding to the phosphothreonine or phosphoserine motifs in the targets [13]. Therefore, it was believed that the interaction of ACS and 14-3-3, if it did widely exist in ACC synthases, also occurred in a similar phosphorylation-dependent manner. To date, two phosphorylated sites in the C terminus of ACS have been uncovered. Tatsuki and Mori reported that the serine of RXSX of both type 1 and 2 was shown to be phosphorylated by an unidentified calcium-dependent protein kinase (CDPK) from wounded fruits and the phosphorylation could be blocked by chelation with EGTA [9]. Liu and Zhang demonstrated that MPK6 could phosphorylate three serine residues in the tails after the RXSX motif of type 1 ACSs [12]. However, the phosphorylation of RXSX might not be excluded since EGTA was used in the buffer of the protein kinase assay [12]. These results indicated that type 1 ACC synthases were under the regulation of two kinase pathways, MPK6 cascade and an unidentified CDPK pathway, and hence two potential 14-3-3 binding sites resided in the C terminus of type 1 ACSs. On the other hand, the MPK6 phosphorylated sites were absent in type 2 ACSs due to a lack of the short tail sequence after RXSX, while the RXSX motifs were conserved in both types of isozymes. Considering this conservation and the possibility that 14-3-3s bind to both types of ACSs, we tended to believe that the phosphorylated RXSX motif was a potential 14-3-3 binding site and that it mediated the interactions of 14-3-3 and ACSs *in vivo*.

Although 14-3-3 proteins exhibit high affinity for two motifs: motif I, R(S/Ar)XpSXP, and motif II, RX(Ar/S)XpSXP (pS denotes pSer/pThr and Ar denotes aromatic residues) [22], binding targets also have been identified that do not contain both of these motifs. Intriguingly, we also found a conserved 14-3-3-binding motif I, RSVSVP, before and very near to the RXSX domain in OsACS1. However, the conserved motif was absent from most plant ACSs when we screened with Scansite. Therefore, it is more likely that 14-3-3 recognizes a non-conserved binding motif RXSX in the C terminus of ACS rather than conserved 14-3-3 sites. Studies using the truncated or site mutated forms of ACS would be needed to accurately identify the 14-3-3 binding sites.

Recent studies have shown that the phosphorylation of ACC synthases by CDPK or MPK6 seemed to help the accumulation of ACS proteins but have almost no effect on enzyme activity, at least in *in vitro* enzymatic assays [9, 12]. Additionally, ETO1 protein identified from the *Arabidopsis eto1* mutant has been proven to inhibit enzyme activity *in vitro* and promote AtACS5 degradation by the 26S proteasome-dependent pathway [5]. AtACS5 belongs to type 2 ACSs and its truncated form, with a deletion of the C-terminal 12 residues containing RXSX motif, was unable to interact with ETO1 [5, 7]. Interestingly, in another overproduced ethylene mutant, *eto3*, the valine residue (V) in the WVFRVSW sequence was mutated to aspartic acid (D) in AtACS9 (which is the closest relative of AtACS5 and also belongs to type 2 ACSs) resulting in an overproduction of ethylene [6]. It should be noted that the residue change from V to D probably mimics the phosphorylation modification, protecting AtACS9 from the fate of binding with ETO1 and degradation. These results indicated that the phosphorylation of the serine in WVFRVSW might play an essential role in the regulation of ACS stability.

A new hypothesis to address the interaction between 14-3-3 and ACS. On the basis of these experimental data, we propose a hypothesis to elucidate the possible biological function of the interaction of 14-3-3 and ACS (Fig. 3). In normal conditions, ETO1 constitutively interacts with ACS and causes two possible effects: directly inhibiting the enzyme activity and/or linking the enzyme to the 26S proteasome-dependent protein degradation pathway. When plants suffer from environmental assaults and/or grow under a specific developmental stage, a higher level

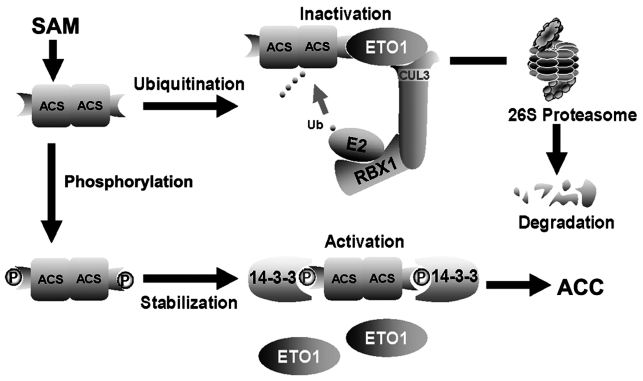

Fig. 3. Model for specific regulation of ACS activity. The C-terminus of ACS is drawn as a small extruding cylinder. Note that each pair of ACS forms a dimer. ACS dimers interact with 14-3-3 or ETO1 proteins, depending on growth conditions. As a result, the ACS is inhibited in enzymatic activity and targeted for proteasome-dependent degradation when it binds to ETO1. However, phosphorylation (circles with letter P) of the C-termini of ACS may promote interaction between ACS and 14-3-3 and may inhibit binding to ETO1 proteins, resulting in ACC synthesis.

of ethylene is needed. In this case, the serine of RXSX is phosphorylated, resulting in a conformational change in the C terminus and subsequent 14-3-3 binding. As a consequence, the binding of ETO1 to ACS is hindered and/or the dephosphorylation of ACS is prevented, which then causes an accumulation of the functional ACSs and a higher yield of ethylene gas. This regulation of ACS stability through 14-3-3 binding might be conserved in type 1 and 2 ACSs since the RXSX motif is conserved in both types. This new hypothesis provides more insight into the regulatory mechanisms of ACS activation mediated by 14-3-3 proteins.

As for type 3 ACSs, a distinct regulation might exist because of the lack of all known phosphorylation sites in their C terminus. Moreover, the study performed by Yoshida et al. showed that AtETO1 specifically interacted with tomato type 2 but not with type 1 ACSs in a yeast two-hybrid assay [10], whereas the interaction between ETO1 and type 1 ACSs could not be still excluded since only a heterogeneous ETO1 and yeast system were used in their study. Even if type 1 ACSs cannot be recognized by ETO1 protein, it is still possible that other ETO1-like adapter proteins bind to type 1 ACSs and perform a similar function because this type of ACS is also degraded in normal conditions and stabilized after phosphorylation. However, the existence of MPK6 sites in type 1 ACSs makes the problem more complex, which could suggest that type 1 ACSs might undergo more complicated regulation than the current model we proposed.

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