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Article Addendum

Arabidopsis ACBP6 is an acyl-CoA-binding protein associated with phospholipid metabolism

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In our recent paper in Plant Physiology, we showed that the *Arabidopsis thaliana* 10-kD acyl-CoA-binding protein, ACBP6, is subcellularly localized to the cytosol and that the overexpression of ACBP6 in transgenic Arabidopsis enhanced freezing tolerance. ACBP6-conferred freezing tolerance was independent of induced cold-regulated (*COLD-RESPONSIVE*) gene expression, but was correlated to an enhanced expression of phospholipase Dδ (PLDδ). Lipid analyses on cold-acclimated freezing-treated ACBP6-overexpressors revealed a decline in phosphatidylcholine (PC) and an elevation of phosphatidic acid (PA) in comparison to wild type. Furthermore, the His-tagged ACBP6 recombinant protein was observed using in vitro filter-binding assays to bind PC, but not PA or lysophosphatidylcholine. Taken together, our results implicate roles for ACBP6 in phospholipid metabolism that is related to gene regulation and PC-binding/transfer. This represents the first report demonstrating the in vitro binding of an ACBP to a phospholipid. The effect of ACBP6 on *PLD*δ expression is reminiscent of yeast 10-kD ACBP function in the regulation of genes associated with stress responses, fatty acid synthesis and phospholipid synthesis. However, the yeast ACBP regulates the expression of genes involved in phospholipid synthesis by donation of acyl-CoA esters and its binding to phospholipids remains to be demonstrated.

Arabidopsis Encodes a Family of Acyl-Coenzyme A-Binding Proteins

Acyl-CoA-binding proteins (ACBPs) show conservation at an acyl-CoA-binding domain which can potentially bind long-chain acyl-CoA esters. A 10-kD form of ACBP was first identified from rat brain.[1] Subsequent investigations on mammalian 10-kD ACBPs indicated functions related to acyl-CoA transport, protection of cytosolic acyl-CoAs from hydrolysis by cellular acyl-CoA hydrolases, maintenance of intracellular acyl-CoA pools, and gene regulation.[2] Other than the 10-kD ACBP, much larger forms of ACBPs have been reported in *A. thaliana*,[3-5] *Caenorhabditis elegans*[6] and *Cryptosporidium parvum*.[7]

In Arabidopsis, six genes have been identified to encode ACBPs and they have been designated *ACBP1* to *ACBP6*.[5] ACBP1 and ACBP2 are membrane-associated proteins, ACBP3 is extracellularly-targeted, and ACBP4 and ACBP5 contain kelch-motifs.[5] Acyl-CoA-binding domains in ACBP1 to ACBP6 have been shown to bind long-chain acyl-CoA esters in vitro, with varying affinities, suggestive of their non-overlapping roles in plant lipid metabolism.[3-5,8,9] *C*-terminal ankyrin repeats in ACBP1 and ACBP2 and kelch motifs in ACBP4 and ACBP5, can potentially mediate protein-protein interactions, while *N*-terminal transmembrane domains in ACBP1 and ACBP2 target them to the endoplasmic reticulum (ER) and the plasma membrane.[5,10-12]

Expression of ACBP6 is Cold-Inducible and ACBP6-overexpressors are Conferred with Freezing Tolerance

The subcellular localization of Arabidopsis ACBP6 to the cytosol was confirmed by analyses of transgenic Arabidopsis expressing autofluorescence-tagged ACBP6, and by western blot analysis of subcellular fractions using ACBP6-specific antibodies. Subsequently, the expression of *ACBP6* in response to stress was investigated, because its homologues in cucumber, pumpkin and rice have been suggested to function in acyl-CoA transport, stress and/or defense.[13,14] Results, from both northern blot and western blot analyses, revealed that ACBP6 expression is cold (4°C)-inducible. Consistently, the *acbp6* knockout mutant showed an enhanced sensitivity to freezing treatment (-8°C), and transgenic Arabidopsis overexpressing ACBP6 were conferred freezing tolerance.

ACBP6-overexpressors showed an enhanced expression of the gene encoding phospholipase PLDδ, mimicking PLDδ-overexpressors that were also observed freezing-tolerant.[15] Elevated expression of *PLD*δ in ACBP6-overexpressors occurred during non-acclimation, cold acclimation, freezing and thawing stages. In contrast, the *acbp6* mutant showed decreased expression at these stages. Since 10-kD ACBPs are highly conserved across species, perhaps some functions inclusive of gene regulation and maintenance of intracellular cytosolic lipid pools, are retained amongst 10-kD eukaryotic ACBPs. The effect of ACBP6 on *PLD*δ expression is reminiscent of its yeast homologue which is

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involved in the regulation of genes associated with stress responses, fatty acid synthesis and phospholipid synthesis.[16]

Role for ACBP6 in Phospholipid Metabloism

Following freezing treatment, PLDδ-overexpressors showed decreases in phospholipid species, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), and increases in their metabolites, phosphatidic acid (PA) and lysophospholipids.[17] Lipid profiling of cold-acclimated ACBP6-overexpressors following freezing treatment also revealed that they accumulated less PC than wild type. In contrast, the PA content increased in both genotypes, with greater accumulation in ACBP6-overexpressors. Decline in species of 34:3 PC, 34:2 PC, 36:6 PC, 36:5 PC, 36:4 PC, 36:3 PC and 36:2 PC corresponded well to elevation in PA species, suggesting that the PA accumulation was primarily derived from PC.

Using in vitro filter-binding assays, recombinant His-tagged ACBP6 was shown to bind 16:0-PC, 18:0-PC, 18:1-PC and 18:2-PC, but not 14:0-PC, PA or LysoPC, further implicating ACBP6 in phospholipid metabolism in Arabidopsis. Our findings represent the first report demonstrating binding of an ACBP to a phospholipid in vitro. Although the 10-kD yeast ACBP is known to function in phospholipid metabolism, and has been shown to regulate the expression of genes in phospholipid synthesis by donation of acyl-CoA esters, it has not been demonstrated to bind any phospholipids.[16]

Conclusions and Perspectives

Oleoyl-CoAs arising from de novo fatty acid biosynthesis in the chloroplasts need to be transported to the ER for the "eukaryotic pathway" in lipid metabolism. Since His-tagged ACBP6 binds oleoyl-CoA with relatively lower affinity than His-tagged ACBP4 and ACBP5, ACBP4 and ACBP5 (rather than ACBP6) are likely to play more significant roles in oleoyl-CoA transfer from the chloroplasts to the ER (Chye ML and Xiao S, unpublished data). Here, we show that ACBP6 has a role in phospholipid metabolism related to mediating freezing stress responses, and possibly in the cytosolic trafficking of PC. Previously, we have demonstrated that ACBP1 and ACBP2, but not ACBP6, are responsive to heavy metal (lead) stress.[18]

In plant cells, the main site for phospholipid synthesis is the ER.[19] In order to maintain a stable phospholipid composition in different cellular membranes, continuous exchange of phospholipids in the cellular membranes occurs.[20] Transfer of ER-derived PC via the cytosol to the choroplast is a prerequisite for the utilization of extraplastidial PC for the biogenesis of the plastidial outer envelope membrane.[21] Investigations from non-plant sources suggest that phospholipid transfer from the ER to other intracellular membranes are mediated by cytosolic lipid transfer proteins and currently little is known of such proteins operating in plants.[20,22-24] Since ACBP6 can bind PC in vitro, we propose that ACBP6 is a possible candidate in the cytosolic binding and trafficking of PC in plant cells. The in vivo lipid-transfer activity of ACBP6 and its significance in PC-trafficking in planta await further investigations using in vivo and in vitro experiments.

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