ARTICLE ADDENDUM

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Is AKR2A an essential molecular chaperone for a class of membrane-bound proteins in plants?

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The Arabidopsis ankyrin-repeat containing protein 2A (AKR2A) was shown to be an essential molecular chaperone for the peroxisomal membrane-bound ascorbate peroxidase 3 (APX3), because the biogenesis of APX3 depends on the function of AKR2A in plant cells. AKR2A binds specifically to a sequence in APX3 that is made up of a transmembrane domain followed by a few positively charged amino acid residues; this sequence is named as AKR2A-binding sequence or ABS. Interestingly, a sequence in the chloroplast outer envelope protein 7 (OEP7) shares similar features to ABS and is able to bind specifically to AKR2A, suggesting a possibility that proteins with a sequence similar to ABS could bind to AKR2A and they are all likely ligand proteins of AKR2A. This hypothesis was supported by analyzing 5 additional proteins that contain sequences similar to ABS using the yeast two-hybrid technique. A preliminary survey in the Arabidopsis genome indicates that there are at least 500 genes encoding proteins that contain sequences similar to ABS, which raises interesting questions: are these proteins AKR2A's ligand proteins and does AKR2A play a critical role in the biogenesis of these proteins in plants?

Key words: Arabidopsis, membrane protein, molecular chaperone, protein targeting, transmembrane domain

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The Arabidopsis ankyrin-repeat containing protein 2A (AKR2A) is an essential molecular chaperone for the peroxisomal membrane-bound ascorbate peroxidase 3 (APX3).[1] Both AKR2A and APX3 were identified as GF14λ-interacting proteins[2,3] when the mode of action of a 14-3-3 protein, GF14λ[4] was studied. In characterizing the enzymatic property of APX3, there was some initial difficulty in purifying the expressed APX3 from a bacterial expression system. Although APX3 could be expressed in *E. Coli* cells in large quantities, as evidenced by directly boiling the bacterial cells and analyzing the bacterial cells by SDS-PAGE and Western blot analysis (**Fig. 1**), APX3 enzymatic activity in the supernatant fraction was not detectable after cells were broken by sonication (**Fig. 1**). The reason that APX3 activity was not detectable in the supernatant fraction was likely caused by the transmembrane domain that occurs at the C-terminal end of APX3; because these hydrophobic domains could interact with one another, forming insoluble aggregates in bacterial cells. When a truncated APX3 was expressed, i.e., APX3 without the transmembrane domain (APX3Δ in **Fig. 1**), APX3 activity was then detectable in the supernatant fraction of bacterial cellular extracts. If a protein is able to bind to APX3's transmembrane domain immediately after or during translation of APX3, this protein could prevent APX3 from forming insoluble aggregates among themselves. APX3 activity would then be detectable in the supernatant fraction. Because some 14-3-3-interacting proteins were shown to interact with one another,[5] the best candidate that could interact with APX3 should be AKR2A (because they are both GF14λ-interacting proteins). This possibility was tested by simultaneously expressing both *APX3* and *AKR2A* in the same bacterial cell; APX3 activity was indeed detectable in the supernatant fraction of bacterial cellular extracts (**Fig. 1**).

This was the first evidence that AKR2A interacts with APX3 and the interaction

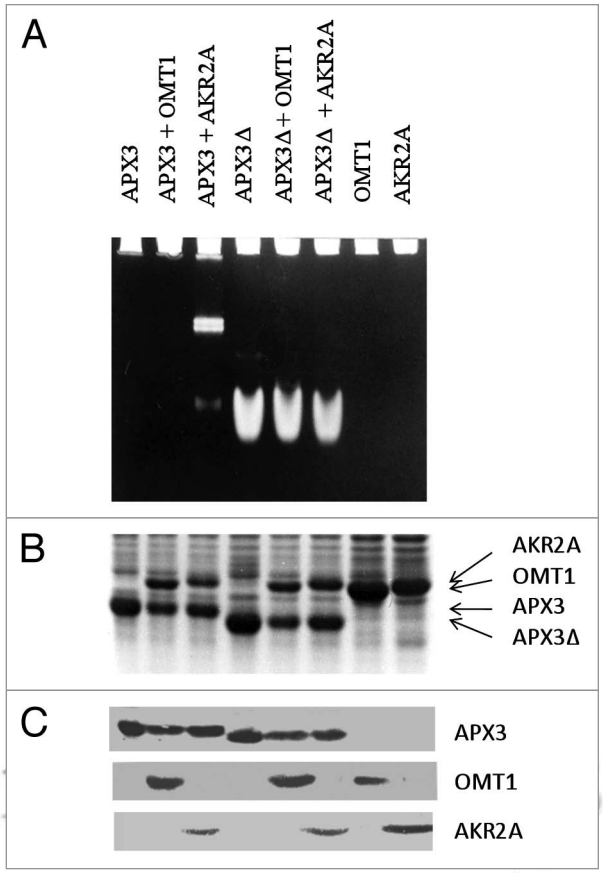

Figure 1. Protein-protein interaction between AKR2A and APX3 in bacterial cells. (A) Analysis of APX3 activity in supernatant fractions of various bacterial cells. In lanes, APX3, supernatant from cells that express full-length APX3; APX3 + OMT1, supernatant from cells that express both full-length APX3 and OMT1 (*O*-methyltransferase1,[7]); APX3 + AKR2A, supernatant from cells that express both full-length APX3 and AKR2A; APX3Δ, supernatant from cells that express a partial APX3 (i.e., lacking the transmembrane domain and the last seven amino acid residues); APX3Δ + OMT1, supernatant from cells that express both APX3Δ and OMT1; APX3Δ + AKR2A, supernatant from cells that express both APX3Δ and AKR2A; OMT1, supernatant from cells that express OMT1; AKR2A, supernatant from cells that express AKR2A. The white bands in the gel represent APX3 activities as assayed by using the method of Mittler and Zilinskas.[8] (B) Bacterial cells expressing various target proteins were analyzed directly by using SDS-PAGE method and the positions of the expressed target proteins are marked on the right. (C) Bacterial cells expressing various target proteins were analyzed by western blot. The antibodies used are listed on the right.

site involves the C-terminal transmembrane domain of APX3. To further define the amino acid residues involved in the AKR2A-APX3 interaction, yeast two-hybrid experiments were conducted with various deletion fragments of AKR2A and APX3.[1] It was found that in addition to the transmembrane domain, the positively charged amino acid residues following the transmembrane domain also play a role in the AKR2A-APX3 interaction.[1] This sequence in APX3 was designated as AKR2A-binding sequence (ABS). In order to understand the biological function of the AKR2A-APX3 interaction, several *akr2a* mutants that displayed reduced or altered interaction with APX3 were created and analyzed. Results indicated that reduced AKR2A activity leads to severe developmental, phenotypic, and physiological abnormalities including reduced steady-state level of APX3 and reduced targeting of APX3 to peroxisomal membranes in Arabidopsis.[1] The pleiotropic nature of *akr2a* mutants indicated that AKR2A plays more roles in addition to chaperoning APX3. Indeed this work was corroborated by a finding that AKR2A is also required for the biogenesis of the chloroplast outer envelope protein 7 (OEP7).[6] More importantly, the interaction between AKR2A and OEP7 also involves a sequence in OEP7 that is similar to the ABS found in APX3.

There is no apparent similarity, at the amino acid level, between the sequences of the AKR2A-binding site found in APX3 and OEP7; it appears that what AKR2A recognizes in its ligand proteins is the structural feature: single transmembrane domain followed by one or a few positively charged amino acid residues. Therefore, these AKR2A-binding sequences should all be designated as ABS, and it was predicted that any protein with an ABS could be AKR2A's interacting protein. Five such proteins, APX5, TOC34, TOM20, cytochrome b_5 (CB5) and cytochrome b_5 reductase (CB5R) were tested, and indeed all five proteins interacted with AKR2A in the yeast two-hybrid system.[1] More importantly, the interaction sites of these proteins are their ABS in every case tested.[1] Based on these discoveries, it is proposed that AKR2A is a molecular chaperone for this group of ABS-containing proteins.

Among the seven AKR2A-interacting proteins that were characterized, the ABS is found at C-terminal end of four proteins (APX3, APX5, CB5 and TOM20), near N-terminal end of two proteins (OEP7 and CB5R), and near C-terminal end of one protein (TOC34), suggesting that the position of ABS in these membrane proteins does not affect its interaction with AKR2A. Furthermore, in all cases, AKR2A binds to its ligand proteins that contain only one ABS. AKR2A does not appear to bind to proteins that contain multiple transmembrane domains such as PMP22,[1,6] even though these transmembrane domains are followed by a few positively charged amino acid residues.

APX3 and APX5 are peroxisomal membrane-bound, OEP7 and TOC34 are chloroplast outer envelope proteins, TOM20 is a mitochondrion outer membrane protein and CB5 and CB5R are microsomal membrane (ER-membrane) proteins. Therefore, AKR2A is clearly not responsible for targeting these proteins to their specific membranes; instead AKR2A serves as a molecular chaperone to prevent these proteins from forming aggregates through their hydrophobic domain in ABS after translation (**Fig. 2**). Perhaps, AKR2A's binding to the ABS of these membrane proteins also keeps

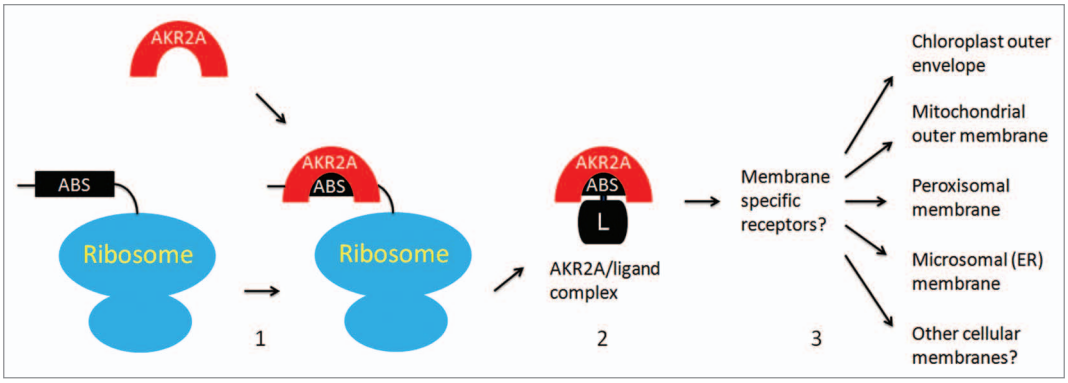

Figure 2. Model on how AKR2A chaperones its ligand proteins. (1) AKR2A binds to ABS of a nascent protein that is being synthesized from a free ribosome. (2) AKR2A keeps its ligand protein (L) in the cytoplasm. (3) With the help of membrane-specific receptors, AKR2A's ligand proteins are sent to their specific membranes.

these proteins in insertion competent state before they are sent to their specific destinations. It is clear that other factors, such as organellar membrane-specific receptors, must be required for sending these proteins to their specific membranes (**Fig. 2**).

The Arabidopsis proteome was analyzed and it was found that there are at least 500 proteins that contain sequences similar to ABS (http://bio.scu.edu.cn/list.xls). Would these proteins be AKR2A's ligand proteins? Some of them, if not all, might be, but it will be a challenging task to experimentally test these proteins one by one. A better bioinformatics tool that can provide clues on the mode of action of the protein-protein interactions between AKR2A and its known ligand proteins should help us designing next set of experiments in order to answer the above question in an efficient way.

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