学位論文の要旨

Abstract of Thesis

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学位論文題目 Title of Thesis（学位論文題目が英語の場合は和訳を付記）

Structural insights into a citrate transporter that mediates aluminum tolerance

（和訳：アルミニウム耐性に関わるクエン酸輸送体の構造的知見）

学位論文の要旨 Abstract of Thesis

Soil acidification affects over one-third of the world's arable land, primarily due to natural processes and anthropogenic activities such as excessive nitrogen-based fertilization and intensive agriculture. In acidic soils, aluminum exists in an ionized form of Al^{3+}, which inhibits root growth and poses a serious threat to crop productivity. Barley (*Hordeum vulgare, Hv*), one of the most Al^{3+}-sensitive crops, mitigates this toxicity by secreting citrate from its roots to chelate Al^{3+} in Al-tolerant varieties. This secretion is mediated by HvAACT1, a member of the multidrug and toxic compound extrusion (MATE) family. *HvAACT1* is constitutively expressed at the root tips and is induced upon Al exposure. Despite its functional importance, the structural basis of citrate transport by HvAACT1 remains poorly understood.

To date, structures of several members of the MATE family belonging to all three subfamilies – NorM, DinF, and eukaryotic MATE – have been reported. However, all these transporters transport cationic or polyaromatic substrates. The transport mechanism for negatively charged substrates, such as citrate, cannot be inferred from the available structures. Therefore, this thesis aims to elucidate the crystal structure of HvAACT1 and provide structural insights into its mechanism of citrate transport.

This study reports the optimization of the HvAACT1 protein construct for crystallization, resulting in the mutant $HvAACT1_{cryst}$, by deleting two disordered regions and introducing five-point mutations to increase the thermostability and reduce surface entropy. Functional assays confirmed that $HvAACT1_{cryst}$ still retains citrate export activity in *Xenopus* oocytes. Thus, the data obtained from $HvAACT1_{cryst}$ are sufficient to elucidate the mechanism of citrate efflux of the wild-type. Further optimization of crystallization conditions, particularly through additive screening and detergent exchange, enabled the production of a crystal that yielded X-ray diffraction data at a resolution of 3.2 Å.

The crystal structure reveals that $HvAACT1_{cryst}$ adopts an outward-facing conformation, characterized by an open extracellular gate and a closed intracellular gate. The tight closure of the intracellular gate observed in the crystal structure is stabilized by salt bridges and hydrogen bonds formed between the residues located at the interface of the N- and C-terminal domains. Electrostatic surface

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potential of HvAACT1$_{cryst}$ revealed that the inner cavity in its N-terminal domain is mainly positively charged, while that in the C-terminal domain is primarily negatively charged. This charge distribution contrasts with that observed in other plant MATE transporters, suggesting divergence in the underlying transport mechanism. A closer structural examination revealed a putative citrate-binding site, which is formed by three conserved basic residues in the C-terminal domain. This binding site is distinct from the binding sites that are made of acidic residues observed in other MATE transporters. Inward-facing AlphaFold2 predicted structure of HvAACT1$_{cryst}$ suggested that this putative binding site can be accessed from the intracellular side with a rigid body movement of the N-terminal and C-terminal domains.

The N-terminal domain of HvAACT1$_{cryst}$ harbors two potential proton binding sites, one of which corresponds to the conserved and critical Asp pair found in the DinF subfamily. Structural coupling between the distinct substrate- and proton-binding sites appears to be mediated by an extended hydrogen-bonding network in the extracellular half of the N-terminal domain, resembling that of DinF transporters. A conserved proline residue likely imparts conformational flexibility to the transmembrane helix-1 in the N-terminal domain, which is essential for its functionality. These structural proposals were verified by functional analysis of HvAACT1 mutants, where alanine substitutions at putative substrate or proton binding sites, as well as within the hydrogen-bonding network, resulted in significantly reduced citrate efflux compared to the wild type. Results of molecular dynamics simulations using the inward-facing predicted model of HvAACT1$_{cryst}$ revealed that one of the three basic residues consistently interacted with citrate. In contrast, citrate alternated between the putative substrate binding site and the vicinity of the putative protonation site, supporting the functional relevance of this substrate binding site. Furthermore, phylogenetic analysis indicates that citrate-transporting MATE proteins are evolutionarily related to the DinF MATE subfamily and have uniquely evolved in the plant kingdom to transport negatively charged substrates.

In conclusion, the structural insights into HvAACT1-mediated citrate transport presented in this thesis provide a molecular basis for aluminum tolerance in crops and shed light on the evolutionary diversification of substrate specificity within the MATE transporter family.