論文の内容の要旨

Improving cold-activity of phytase by empirical and rational design

(経験的および合理的設計によるフィターゼの低温高活性化)

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【Background and purpose】

Phosphorous is one of the essential elements for plant growth. However, the phosphorous in the fertilizer tends to be accumulated as a form called organic phosphorous, which is not available for plants. Among different kinds of organic phosphorous, phytic acid is the most abundant, comprising up to 90% of the total organic phosphorous. By

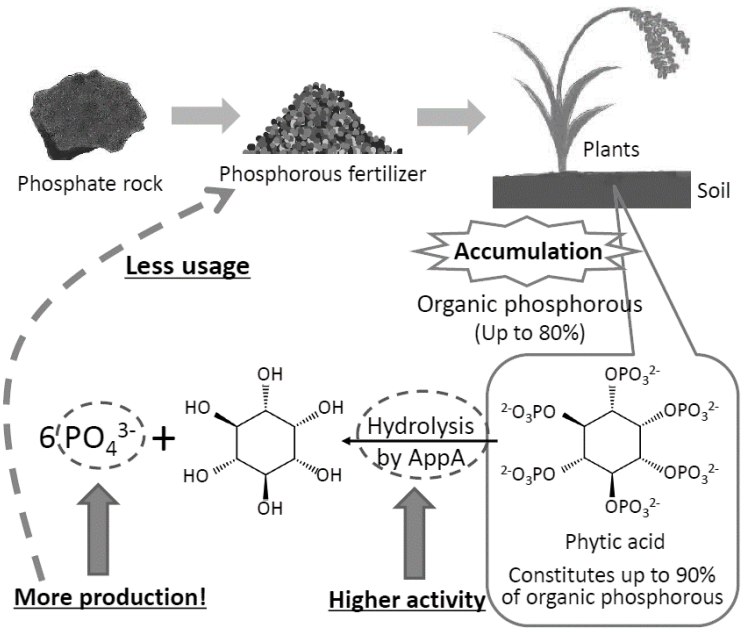

Fig. 1 Image abstract of this thesis

extracting phosphorous efficiently, the phosphorous depletion problem might be overcome.

The category of enzymes that are able to take phosphorous out of phytic acid is called phytases. In this thesis, a phytase from *E. coli*, AppA, was chosen for its high activity. However, under the relatively low soil temperature (15°C) of Japan, AppA can only express less than 20% of its best activity. Hence, this thesis aimed to increase the cold activity of phytase AppA, specifically at 15°C.

【Methods】

Utilizing three different strategies, enhancement of AppA cold activity was attempted.

(i) **Improving AppA cold-activity by directed evolution**

After establishing the AppA expression system in *E. coli*, AppA mutants went through two steps of selection. The first step involved the colony-based AppA enzyme assay on filter paper. If a mutant has AppA activity, a blue spot will appear on the filter paper after the color development process. By the ImageJ software, the blue spot area was divided by the colony area, and the mutants with higher values proceeded to the next selection. The second step is performed by cultivating selected mutants and wild type, and carrying out AppA enzyme assay and SDS-PAGE assay using cell lysate. The AppA activity was standardized by dividing with AppA band intensity.

(ii) **Rational design 1: Ala/Gly scanning of active site loop and substitution of less-affected sites**

This strategy started with first determining the crucial amino acids for AppA

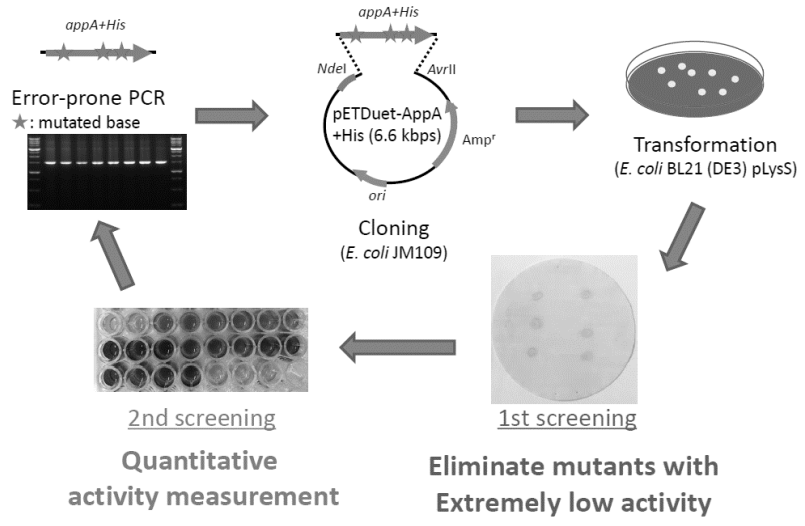

Fig. 2 Constructed AppA screening system

function at its optimum temperature, 60°C, by performing Ala/Gly scanning mutagenesis of the active site loop (R16-T26). Then, those mutants that were less affected by Ala/Gly mutations were chosen and further mutated.

(iii) **Rational design 2: Replacement of residues on helices**

Based on a previous statistical study of cold-adaptation by enzymes, some residues were picked up and mutated with frequently observed residues in cold-adapted enzymes.

【Results and Discussion】

(i) AppA expression system in *E. coli* BL21(DE3)pLysS was constructed. AppA mutants were prepared by introducing random mutations by error-prone PCR. Mutant colonies were used to undergo the first screening, and 63 mutant colonies were chosen out of more than 800 candidate colonies. The 63 mutants and wild type AppA were cultivated, and AppA enzymatic assay and SDS-PAGE analysis using cell lysate and pellet samples were carried out. After the AppA enzyme assay, the mutants with activity higher than wild type in enzyme unit (U) were chosen and standardized by AppA band intensity from SDS-PAGE analysis. Among the 18 mutants analyzed, mutant 3 showed approximately 1.6-times as much activity of wild type AppA.

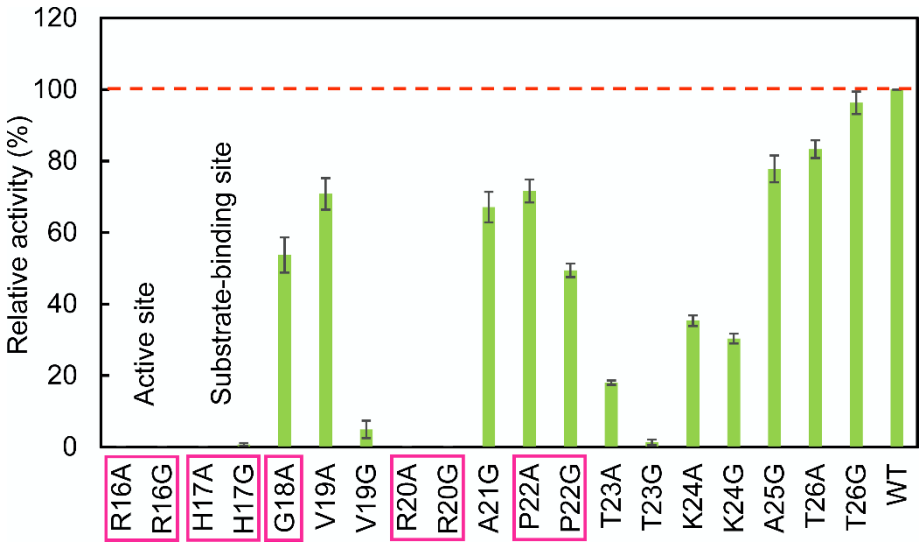

Fig. 3 Relative activity of Ala and Gly mutants of active site loop and wild type AppA.

(ii) Ala/Gly scanning mutagenesis to AppA active site loop revealed the contribution of each residue to AppA activity. The enzyme assay reinforced the essential functions of charged conserved sites (R16, H17, and R20) for the AppA catalysis. Although not conserved, T23 and K24 mutants lost a large fraction of activity. T23 mutants lost more activity than K24 mutants, possibly because of a water-mediated contact with the substrate. Based on these results, the residues that were less affected by Ala/Gly mutations were mutated.

(iii) Residues on helices were mutated to residues popular in cold-adapted enzymes. Although the mutation sites were selected based on a specific condition, the surrounding environment of the residues apparently affected the mutation effect. For example, one mutant retained roughly 60% relative activity while another mutant exhibited almost 200% activity compared to the wild type.

Future perspectives: Combinatorial mutants will be constructed for a higher cold activity. Also, the second round of directed evolution would be carried out to obtain mutants that cannot be obtained through the rational design. Finally, a detailed biochemical study on cold-active AppA mutants will be performed using the purified samples.