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Article

Complementation of *E. coli cysQ* Mutant with *Arabidopsis AHL* Gene Encoding a 3'(2'),5'-Bisphosphate Nucleotidase

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Arabidopsis *AHL* gene encodes a 3'(2')-phosphoadenosine 5'-phosphate (PAP)-specific phosphatase that plays a role in the sulfate activation pathway. We complemented *E. coli cysQ* mutant defective in cysteine biosynthesis with the *AHL* gene. *AHL* cDNA was cloned into the prokaryotic expression vector pKK388-1 and transformed into the bacterial mutant. Since *cysQ* mutant is a leaky cysteine auxotroph only under aerobic conditions, the bacteria were grown in liquid media with vigorous shaking to provide more aeration. In cysteine-free medium, *cysQ* mutant and the mutant harboring empty vector did not grow well, whereas cells harboring *AHL* cDNA exhibited significantly improved growth with doubling time of approximately 3 h. *cysQ* is known to encode a 3'(2'),5'-diphosphonucleoside 3'(2')-phosphohydrolase (DPNPase). However, our data suggest that cysQ protein has PAP-specific phosphatase activity in addition to DPNPase activity. Microbial complementation procedure described in this paper is useful for structure-activity studies of PAP-specific phosphatases identified from microbes and plants.

Key words: *Arabidopsis AHL gene, E. coli cysQ mutant, DPNPase, PAP-phosphatase, cysteine-rich defense proteins, reductive sulfate assimilation.*

Sulfur is one of the major elements of sulfur-containing amino acids, sulfated polysaccharides, sulfolipids, and coenzymes.[1,2] In cellular sulfur metabolism, cysteine lies on the major route of inorganic sulfur incorporation into proteins and cysteine-rich peptides. Among the cysteine-rich peptides, glutathione[3] and phytochelatins[4] are the most abundant thiol compounds that play important roles in the detoxification of herbicides in plants. In addition, several families of small cysteine-rich proteins such as thionins and defensins exert antimicrobial activity in plant-pathogen interactions.[6,7] Thus, sulfur metabolism in plants is closely related to various defense mechanisms against abiotic and biotic stresses.[8]

Inorganic sulfate uptaken from environment is chemically inert in living cells under moderate biological conditions. Thus, sulfate must be activated by reduction to sulfide before incorporated into sulfur-containing compounds.[1] In *E. coli* and plants, sulfate is activated via coupling with ATP by ATP sulfurylase to form adenosine 5'-phosphosulfate (APS). A second ATP-coupled activation is conducted by APS kinase that converts APS into 3'-phosphoadenosine 5'-phosphosulfate (PAPS). PAPS reductase then reduces PAPS into sulfite that is further reduced into sulfide by sulfite reductase. Main pathway of the reductive sulfate assimilation and structure of PAPS are depicted in Fig. 1.

PAPS, at high concentrations, exerts toxicity on cells. 3'(2'), 5'-Diphosphonucleoside 3'(2')-phosphohydrolase (DPNPase) has been suggested to catalyze the conversion of PAPS back into APS to avoid accumulation of PAPS. Proteins encoded by *E. coli* gene *cysQ*[9] and rice *RHL*[10] have been found to have DPNPase activity.

When PAPS is reduced into sulfite, 3'-phosphoadenosine 5'-phosphate (PAP) is generated as a by-product. PAP is then hydrolyzed into AMP and inorganic phosphate by a PAP-specific phosphatase, 3'(2'),5'-bisphosphate nucleotidase. Studies have found that the proteins encoded by yeast *HAL2*,[11] *Arabidopsis SAL1*,[12] and Arabidopsis *AHL*[13,14] genes are PAP-phosphatases. The enzyme contributes to the rapid sulfur flux through the assimilation pathway by accelerating the PAPS-utilizing reactions. More importantly, the enzyme has been suggested to control the level of toxic PAP.[15,16]

HAL2, SAL1, RHL, cysQ, and AHL have similar amino acid sequences including two motifs that are conserved in the inositol monophosphatase family. *AHL*, *RHL*, and *SAL1* cDNAs complemented the yeast *HAL2* mutant, *met22*. Moreover, the gene products of *AHL*, *HAL2*, *RHL*, and *SAL1* utilize both PAPS and PAP as substrates *in vitro*. Thus, it is ambiguous whether these proteins belong to the PAP-phosphatase or DPNPase enzyme class.

Bacterial mutant complementation is one of the simplest procedures to demonstrate the enzyme activity of a protein encoded by a particular gene. However, complementation of

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Abbreviations: APS, adenosine 5'-phosphosulfate; DPNPase, 3'(2'),5'-diphosphonucleoside 3'(2')-phosphohydrolase; IPTG, isopropylthiogalactopyranoside; PAP, 3'(2')-phosphoadenosine 5'-phosphate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate.

Fig. 1. Main pathway of reductive sulfate assimilation and chemical structure of PAPS.

the *E. coli cysQ* mutant has been hampered by the fact that the bacterial mutant is a leaky cysteine auxotroph only during aerobic growth.[9] In this study, attempt was made to complement the *E. coli cysQ* mutant with the *AHL* gene by supplying more aeration to the culture.

Materials and Methods

Microbial strain. The *E. coli* mutant *cysQ* 5649 (*cysQ::kan* in David Botstein's 6128 [HfrH *lacZ*(Am*)*, *trp*(Am) *sup*III^{+}]) was kindly provided by Dr. Douglas E. Berg (Washington University School of Medicine, St. Louis, Missouri, USA).

DNA construct and transformation. The Arabidopsis *AHL* cDNA[13,14] fragment was prepared by the polymerase chain reaction (PCR) using a high fidelity *Taq* polymerase (Boeringer Mannheim). The *AHL* cDNA was cloned into the prokaryotic expression vector pKK388-1 (Clontech Inc.) between the *Nco*I and *Eco*RI sites. The *cysQ* mutant cells were transformed with the construct or empty vector by electroporation. Transformed cells were then selected on LB plates containing 50 μg/m*l* each ampicillin and kanamycin. Orientation of the insert was examined by diagnostic restriction enzyme digestions and DNA sequencing.

Complementation experiments. The selected colony was grown in a liquid LB medium, and 5 m*l* saturated suspension was centrifuged at 3,800 rpm for 5 min. Subsequently, the bacterial residue was resuspended in 5 m*l* sterile water (OD_{600} = ~2.0, ~1 × 10^{9}/m*l*). Fifty microliters of the suspension was added to 50 m*l* liquid M9 minimal medium (NH_4Cl and $CaCl_2$ included) containing 1 M $MgSO_4$ and 0.2% glucose. The medium was supplemented with kanamycin (50 μg/m*l*), tryptophan (50 μg/m*l*), and 1 mM IPTG. For the control experiment, 50 μg/m*l* L-cysteine was added to the medium. The bacteria were grown in a rotary shaker (300 rpm) at 37°C, and their growths were determined by measuring the absorbances of the cultures at 600 nm. For solid medium, 1.5% Bacto agar was added to the medium. The bacteria were grown at 37°C for 2 days on the solid medium.

Results

Amino acid sequence homology. The *E. coli* cysQ (GeneID 1037415) and Arabidopsis AHL (GenBank AF016644) amino acid sequences were aligned using the cluster W program (Fig. 2). The BoxShade program was used to highlight identical amino acid residues. The two motifs that are conserved in the inositol monophosphatase family were found in both sequences. The two putative proteins exhibited 29.9% identity and 50.0% similarity in amino acid residues. Comparison of the nucleotide sequences revealed 39.0% homology between the *cysQ* and *AHL* genes (data not shown).

Fig. 2. Amino acid sequence alignment. The Arabidopsis AHL (GenBank AF016644) and *E. coli* cysQ (GeneID 1037415) amino acid sequences are aligned using the cluster W program. Residues shared by the two sequences are shaded. The two motifs that are conserved in the inositol monophosphatase family are boxed.

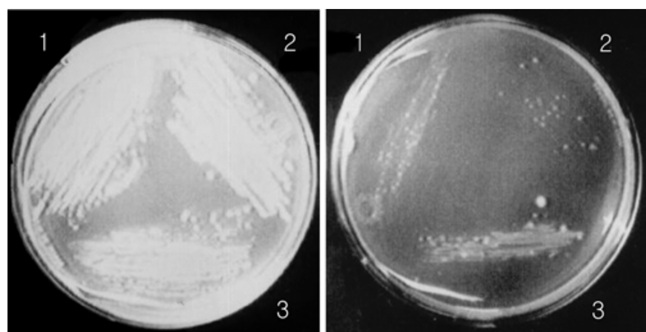

Fig. 3. *cysQ* complementation on solid medium. Saturated bacterial cultures grown in LB medium were washed with sterile water, and 3 μ*l* suspension was streaked on solid (1.5% agar) M9 minimal medium supplemented with kanamycin (50 μg/m*l*), tryptophan (50 μg/m*l*), and 1 mM IPTG (right). For the control experiment, 50 μg/m*l* L-cysteine was added to the medium (left). The bacteria were grown at 37°C for 2 days. *1*, *cysQ* mutant; *2*, *cysQ* mutant harboring the empty vector pKK388-1; *3*, *cysQ* mutant harboring *AHL* cDNA inserted in the pKK388-1 vector.

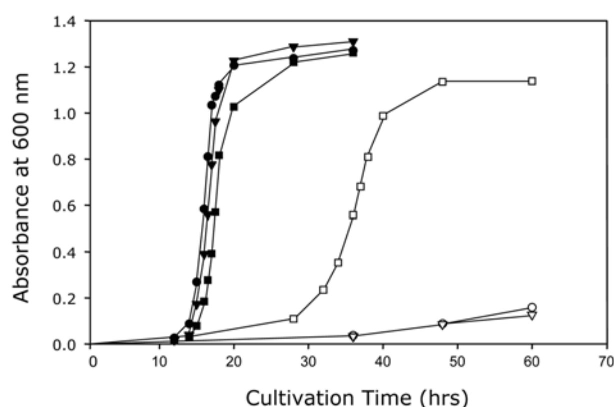

Fig. 4. *cysQ* complementation in liquid medium. Each 50 μ*l* of bacterial suspension was added to 50 m*l* liquid M9 minimal medium without L-cysteine (open symbols). For the control experiment, 50 μg/m*l* L-cysteine was added to the medium (closed symbols). The bacteria were grown on a rotary shaker (300 rpm) at 37°C, and their growths were determined by measuring absorbances at 600 nm. Circles (●, ○), *cysQ* mutant; triangles (▲, △), *cysQ* mutant harboring vector pKK388-1; squares (■, □), *cysQ* mutant harboring vector pKK388-1 containing *AHL* cDNA. Each point in the data represents the average of two replicates.

***cysQ* complementation on solid medium.** To determine whether the protein product of *AHL* gene has a function similar to that of *E. coli* cysQ protein, complementation experiments with bacterial mutants were conducted. When each saturated culture was grown on cysteine-free solid medium (1.5% bacto-agar), the *cysQ* mutant cells harboring *AHL* cDNA grew slightly faster than the mutant cells harboring no or empty vector (Fig. 3). The growth of all strains, however, was indistinguishable after 2-3 days (data not shown). Thus, solid medium appears unable to maintain conditions sufficiently aerobic for growth repression of the *cysQ* mutant cells. A previous study revealed that the *cysQ* mutant exhibits leaky cysteine auxotrophic growth only under aerobic conditions.[9]

***cysQ* complementation in liquid medium.** To provide more aeration, the bacteria were grown in liquid media with vigorous shaking. Under these conditions, the *cysQ* mutant and *cysQ* mutant harboring empty vector did not grow well without the addition of L-cysteine (Fig. 4). In contrast, the cells harboring *AHL* cDNA exhibited significantly improved growth in the cysteine-free media with a doubling time of approximately 3 h. In the media containing L-cysteine (and IPTG), little differences were observed in growth rates between *cysQ* mutant and plasmid-harboring cells, both showing doubling times of approximately 1 h.

Discussion

In this study, the bacterial *cysQ* mutant was successfully complemented with the Arabidopsis *AHL* gene. Solid medium was not adequate for the *cysQ* complementation experiments that require sufficiently aerobic condition for repression of the *cysQ* mutant cell growth. On the other hand, vigorous shaking of the liquid medium provided more aeration. The bacterial cells harboring *AHL* cDNA exhibited significantly improved growth in the cysteine-free medium with a doubling time of approximately 3 h. The newly devised complementation procedure is useful for the structure-function study of the cysQ-like proteins identified from a variety of living organisms.

Previous studies showed that *E. coli* gene *cysQ* encodes a 3'(2'),5'-diphosphonucleoside 3'(2')-phosphohydrolase (DPNPase) that catalyzes the conversion of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) into adenosine 5'-phosphosulfate (APS) in the sulfate activation pathway[9]. In another set of experiments, AHL protein was identified to be a 3'(2')-phosphoadenosine 5'-phosphate (PAP)-specific phosphatase.[14,17] Whereas HAL2, SAL1, and RHL proteins showed the same or higher activity with PAPS compared to PAP,[10-12] AHL enzyme strongly prefers PAP as the substrate.[14,17] Thus, the *AHL*-complementation of *cysQ* mutant indicates that cysQ protein is also a PAP-specific phosphatase, without excluding the possibility that the protein has a DPNPase activity.

PAP-specific phosphatase encoded by the *AHL* gene contributes to the rapid sulfur flux in reductive sulfate assimilation of plants by eliminating PAP, as suggested by studies of similar enzymes in microbial systems. Furthermore, AHL enzyme is one of the initial targets for metal toxicity in plants.[14] Metal sensitivity of the HAL2 nucleotidase is an important determinant of yeast growth at high concentrations of Li^+ and Na^+.[11,15] Indeed, overexpression of *hal2* significantly improved yeast growth at high salt concentrations.[18]

In addition, the PAP-specific phosphatase enzyme activity controls the supply of cysteine, which is a main component of cysteine-rich peptides such as glutathione and phytochelatins that are involved in heavy metal-tolerance of plants.[3,4,19,20] Moreover, the plant defense proteins such as thionine and defensin are highly cysteine-rich.[5-7] Thus, sulfur metabolism

in plants is closely related to various defense mechanisms against abiotic and biotic stresses. Results of this study imply the possibility of generating plants tolerant to high salts or invading pathogens by overexpression of the PAP-specific phosphatase genes, such as *cysQ* and *AHL*.

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