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ORIGINAL RESEARCH PAPER

Complementation of an *E. coli* cysteine auxotrophic mutant for the structural modification study of 3′(2′),5′-bisphosphate nucleotidase

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Abstract The *Arabidopsis AHL* gene encodes a 3′(2′),5′-bisphosphate nucleotidase (BPNTase) involved in the reductive sulfate activation pathway. A bacterial expression vector containing *AHL* cDNA was randomly mutagenized with hydroxylamine and transformed into the *E. coli* cysteine auxotrophic mutant *cysQ*. Bacterial colonies that did not show evidence of complementation, i.e. those that exhibited slower growth on cysteine-free medium, were selected for further study. Sequencing of the *AHL* cDNA in one such clone revealed the conversion of cytosine 635 (C_{635}) to thymine, resulting in an Alanine (A_{212}) to Valine substitution. This microbial complementation procedure is useful in BPNTase structure-activity studies for biotechnological applications.

Keywords Arabidopsis AHL · 3′(2′),5′-bisphosphate nucleotidase (BPNTase) · *E. coli* cysQ · 3′-phosphoadenosine 5′-phosphate (PAP) · Reductive sulfate assimilation

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Introduction

Inorganic sulfate, the major source of sulfur for plants, is chemically inert and thus must be reduced to sulfide in order to be incorporated into sulfur-containing compounds such as cysteine (Leyh 1993). In plants, sulfate is activated via coupling with ATP to form adenosine 5′-phosphosulfate (APS) by ATP sulfurylase, and reduced to sulfite and eventually to sulfide by APS reductase and sulfide reductase (Kopriva 2006; Kopriva and Koprivova 2004). APS can also be phosphorylated to 3′-phosphoadenosine 5′-phosphosulfate (PAPS) by APS kinase, which serves as a source of activated sulfate for a variety of sulfotransferases (Kopriva and Koprivova 2004).

PAPS can be further reduced to sulfite by PAPS reductase (Schmidt and Jäger 1992; Hell 1997), producing 3′-phosphoadenosine 5′-phosphate (PAP) as a byproduct. PAP is then hydrolyzed to AMP and inorganic phosphate by a PAP-specific phosphatase, 3′(2′),5′-bisphosphate nucleotidase (BPNTase). The proteins encoded by *Arabidopsis AHL* (Cheong et al. 1996; Gil-Mascarell et al. 1999), *Arabidopsis SAL1* (Quintero et al. 1996), yeast *HAL2* (Murguía et al. 1995), and human *BPNT1* (Spiegelberg et al. 1999) exhibit BPNTase activity. PAP degradation contributes to the rapid sulfur flux by accelerating PAPS-utilizing reactions. In

addition, the enzyme controls the level of PAP that is toxic to the cell when it is present at high concentrations (Murguía et al. 1996; Dichtl et al. 1997).

BPNTases are sensitive to salt ions (Murguía et al. 1995; Quintero et al. 1996; Gil-Mascarell et al. 1999). In particular, HAL2 has been identified as a target of Na^+ and Li^+ toxicity in yeast (Murguía et al. 1995). Indeed, over-expression of the *HAL2* gene improves the salt tolerance of yeast (Gläser et al. 1993) and tomato plants (Arrillaga et al. 1998). In humans, the clinical effects of lithium and the role of human BPNTase have been investigated to develop pharmacological modulators for the treatment of manic depressive disease (Spiegelberg et al. 2005). As illustrated by yeast HAL2 (Albert et al. 2000), structural studies of BPNTases have become a prerequisite for the biotechnological manipulation of PAP metabolism.

In *E. coli*, 3′(2′),5′-diphosphonucleoside 3′(2′)-phosphohydrolase (DPNPase) catalyzes the conversion of PAPS back to APS to control the cellular PAPS concentration. It has been shown that the proteins encoded by the *E. coli* gene *cysQ* (Neuwald et al. 1992) and the rice gene *RHL* (Peng and Verma 1995) have DPNPase activity.

In this study, we have developed an experimental strategy to identify amino acids critical to the structure and function of BPNTases. Adapting the previous observations that BPNTases exhibit some activity toward PAPS, the *E. coli cysQ* mutant was complemented with a plant BPNTase gene, *AHL*. Amino acids critical for AHL activity were identified from randomly modified *AHL* cDNA, demonstrating the applicability of bacterial complementation testing for BPNTase structure-activity studies.

Materials and methods

Random mutagenesis of *AHL* cDNA

AHL cDNA (Cheong et al. 1996) was inserted into the prokaryotic expression vector pKK388-1 (Clontech) between the *Nco*I and *Eco*RI sites. The DNA construct (1 μg) was reacted with 3 M hydroxylamine in 10 mM sodium phosphate buffer (pH 7.0) overnight at 37°C. The reaction mixture was dialyzed briefly (10–15 min) using a nylon filter membrane floated on sterile water. The dialyzed residue was diluted 10 times with sterile water and used for bacterial transformation.

Bacterial strain and DNA transformation

The *E. coli* mutant *cysQ* 5649 (*cysQ::kan* in [HfrH *lacZ*(Am), *trp*(Am) *sup*III$^+$]) (Neuwald et al. 1992) was kindly provided by Dr. Douglas E. Berg (Washington University School of Medicine). For the bacterial transformation, 5 ng DNA (in 5 μl) were mixed with 100 μl competent *cysQ* mutant cells and incubated at 42°C for 90 s using the $CaCl_2$ method (Sambrook and Russell 2001). Bacterial suspension (200 μl) were plated on LB medium containing ampicillin (50 μg/ml) and kanamycin (50 μg/ml), and incubated overnight at 37°C.

Complementation test

For the complementation test, a bacterial colony was streaked across a 1 cm^2 on solid (1.5% agar) M9 minimal medium (NH_4Cl and $CaCl_2$ included) containing 1 M $MgSO_4$ and 0.2% glucose supplemented with kanamycin (50 μg/ml), tryptophan (50 μg/ml), and 1 mM IPTG, and incubated at 37°C. Alternatively, each colony was grown in liquid LB medium containing kanamycin and ampicillin, and 5 ml of the saturated suspension were centrifuged at 1,600 × *g* for 5 min. The bacterial pellet was then suspended in 5 ml sterile water (OD_{600} = ~2.0, ~1.2 mg cell dry wt/ml), and 50 μl of the suspension were added to 50 ml M9 minimal medium at 37°C, 300 rpm. Bacterial growth was determined turbidimetrically at 600 nm. One optical density unit corresponds to 0.62 g of dry cell weight per liter (Dahlgren et al. 1993). For the control experiments, L-cysteine (50 μg/ml) were added to the media.

Results and discussion

Similarity between BPNTases and DPNPases

Computational alignments using the cluster W program revealed that several BPNTases (AHL, HAL2, and SAL1) and DPNPases (cysQ and RHL) are similar throughout their amino acid sequences, including two motifs that are conserved in the inositol monophosphatase family (Fig. 1). In particular, the primary sequences of the proteins encoded by the *E. coli cysQ* (GeneID 1037415) gene and the Arabidopsis *AHL* (GenBank AF016644) gene exhibited 30% identity and 50% similarity (Table 1). Based on their nucleotide sequences, the two genes are 41% homologous.

A previously reported in vitro phosphatase activity assay revealed that recombinant AHL prefers 3′-PAP (100%) to PAPS (52%) as a substrate (Gil-Mascarell et al. 1999). In contrast, RHL showed high activity towards PAPS (100%) and 3′-PAP (92%), and it complemented the *E. coli cysQ* mutant (Peng and Verma 1995). Since the proteins encoded by *RHL* and *AHL* utilize both PAPS and PAP as substrates, we hypothesized that the *AHL* gene would complement the bacterial *cysQ* mutant.

cysQ complementation with *AHL*

The *cysQ* mutant exhibits leaky cysteine auxotrophic growth only under aerobic conditions (Neuwald et al. 1992); therefore, to provide adequate aeration, the bacteria were grown in liquid media with vigorous shaking. Under these conditions, the *cysQ* mutant did not grow well without the addition of cysteine (Fig. 2). In contrast, cells harboring *AHL* cDNA exhibited significantly improved growth in cysteine-free media, with a

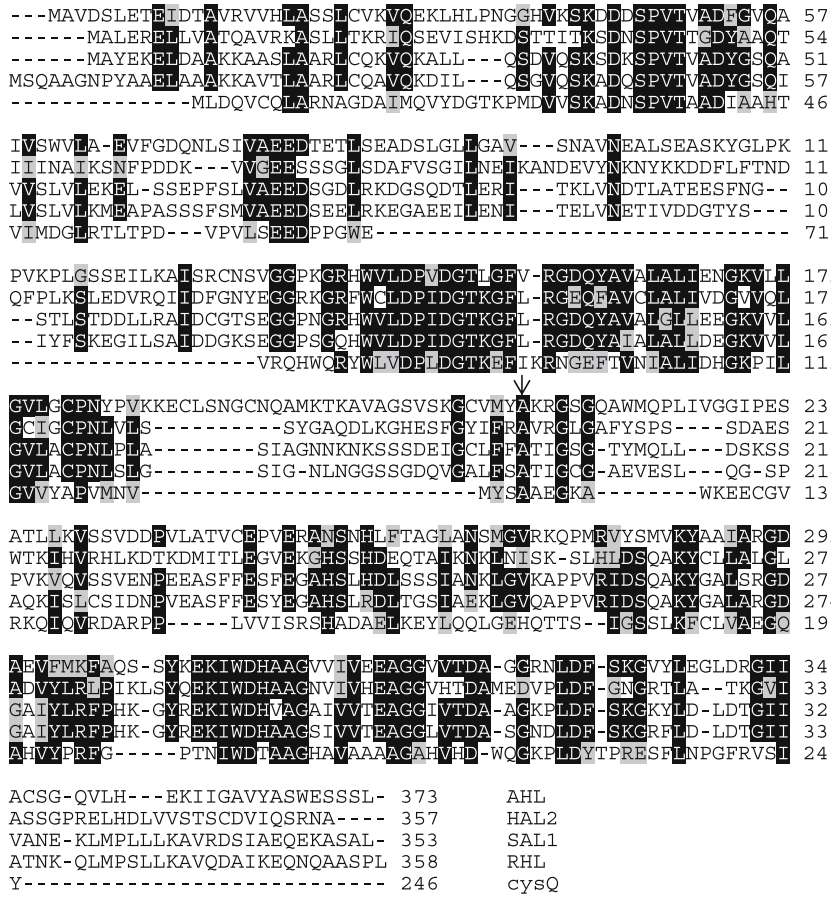

Fig. 1 Amino acid sequence alignment. Deduced amino acid sequences of the *Arabidopsis AHL*, yeast *HAL2*, *Arabidopsis SAL1*, rice *RHL*, and *E. coli cysQ* genes were aligned using the cluster W program. Residues shared by all five sequences are shaded with black (identity) or gray (similarity) background. Arrow indicates Alanine residue that is converted to Valine in *QMF4* insert mutagenized by hydroxylamine treatment (see Fig. 3)

Table 1 Nucleotide and amino acid sequence homologies between BPNTases and DPNPases

	AHL	HAL2	SAL1	RHL	cysQ	
AHL	–	37.6(58.7)	45.4(65.4)	44.7(67.6)	29.9(50.0)	Amino Acid sequence
HAL2	47.6	–	36.2(55.9)	37.8(56.5)	32.5(53.5)	identity (similarity), %
SAL1	53.6	48.3	–	67.5(80.6)	29.9(53.3)	
RHL	52.5	46.8	66.3	–	29.5(49.2)	
CysQ	40.9	42.1	39.1	39.0	–	
	Nucleic acid sequence identity, %					

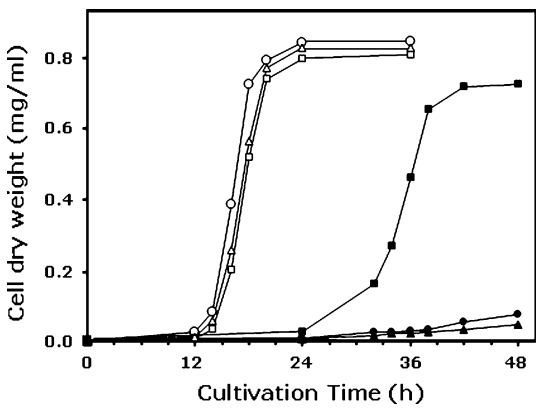

Fig. 2 *E. coli* cysQ complementation. A 50 µl bacterial suspension was added to 50 ml liquid M9 minimal medium supplemented with kanamycin (50 µg/ml), tryptophan (50 µg/ml), and 1 mM IPTG, but without L-cysteine (closed symbols). For the control experiment, L-cysteine (50 µg/ml) were added to the media (open symbols). The bacteria were grown on a rotary shaker (300 rpm) at 37°C. Bacterial growth was determined turbidimetrically at 600 nm and represented as cell dry weight (CDW, OD 1 = ~0.62 mg/ml). Circles (○, ■), the *E. coli cysQ* mutant; squares (□, ■), the *cysQ* mutant harboring pKK388-1 containing *AHL* cDNA; triangles (△, ▲), *QMF4* harboring pKK388-1 containing chemically mutagenized *AHL* cDNA. Each data point represents the average of two replicates

doubling time of approximately 3 h. There was little difference in the growth rates between the *cysQ* mutant and plasmid-harboring cells in media containing L-cysteine and IPTG; both showed doubling times of approximately 1 h.

When each saturated culture was plated on cysteine-free solid medium and incubated overnight, the cells harboring the *AHL* cDNA grew slightly faster than the *cysQ* mutant cells (data not shown). The growth of all strains, however, was indistinguishable after 2 days, indicating that the solid medium was not adequate for *cysQ* complementation, since sufficiently aerobic conditions are required to repress *cysQ* mutant cell growth.

Mutagenesis of *AHL* with hydroxylamine

The *AHL*-pKK388-1 construct was chemically mutagenized using hydroxylamine. Hydroxylamine converts cytosine to thymine, introducing a TA base pair in place of a CG base pair in newly replicated DNA (Hong and Ames 1971). After bacterial transformation with the mutagenized construct, we obtained 2,250 colonies on LB plates containing ampicillin. Each colony was streaked onto cysteine-free solid medium within 1 cm^2 and, after 24 h, those colonies exhibiting slower growth than control bacteria containing untreated *AHL*-pKK388-1 were selected. In total, 76 slow-growing colonies were obtained. Since solid medium was inadequate for the *cysQ* complementation experiments, we retested our colonies in liquid medium for 24 h. Based on the liquid culture test, 11 colonies were identified with an OD_{600} below 0.1, while the control bacteria had an OD_{600} of approximately 0.45.

A second round of transformations was performed to exclude the possibility of chemical damage to other regions of the pKK388-1 vector. The chemically-modified *AHL*-pKK388-1 plasmids were isolated from the 11 bacterial lines and digested with *Eco*RI and *Nco*I. The fragments were extracted from the gel and then inserted into fresh pKK388-1 vector molecules using the original restriction sites and transformed into *cysQ* mutant cells. The new set of bacterial lines was tested for complementation in liquid medium. The results indicated that after 24 h, six bacterial lines, including *QMF4*, showed significantly slow growth (i.e. OD_{600} less than 0.1) in cysteine-free medium (Fig. 2).

Plasmid DNAs were isolated from the six bacterial lines, and the entire inserts were sequenced. In their inserts, one or two TA base pairs were changed to CG base pairs (data not shown). For the bacterial lines that more than two nucleotides were changed in the insert, site-directed mutagenesis should be conducted to confirm the amino acid residue(s) that had influence on the complementation activity.

In the *QMF4* insert, for instance, a cytosine (C_{635}) in *AHL* had been converted to a thymine (Fig. 3). The codon that includes C_{635} (GCA) codes for Alanine (A_{212}) (Fig. 1), while the mutated codon (GTA) codes for Valine. Notably, A_{212} is a highly-conserved residue among BPNTases and DPNPases (Fig. 1). Since both Alanine and Valine are hydrophobic, the effect of this substitution in the primary sequence on the three-dimensional structure of AHL is unclear. The A_{212} of AHL corresponds to the A_{200} of yeast HAL2 that is a constituent of a β-strand located at the end of *N*-terminal domain of the protein (Albert et al. 2000). The active site of HAL2 enzyme lies between *N*-terminal and *C*-terminal domains. It is possible, therefore, that bulky side chain of valine residue influences the structure of the domain-linking region and eventually the active site space. To demonstrate this prediction, further studies, including X-ray crystallography, should be conducted.

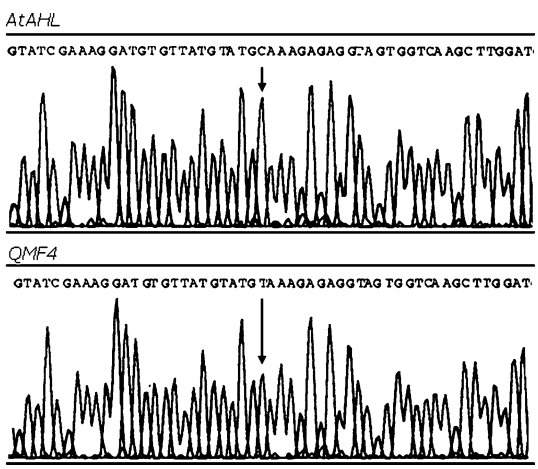

Fig. 3 Partial nucleotide sequences of *AHL* and mutagenized *AHL* (*QMF4*). Plasmid DNA was isolated from each *E. coli* line and the entire inserts were sequenced using an automated sequencer (ABI). Arrows indicate a cytosine (C_{635}) in *AHL* insert and a thymine in *QMF4* insert mutagenized by hydroxylamine treatment

As demonstrated by our experiments, combining random mutagenesis with bacterial complementation is useful for structure-function studies of BPNTases and DPNPases. In addition, our newly-devised method is applicable to the biotechnological manipulation of PAP metabolism in a variety of living organisms, including plants and humans.

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