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Role of OsHAL3 Protein, a Putative 4′-Phosphopantothenoylcysteine Decarboxylase in Rice

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Abstract—In this study, we cloned the *OsHAL3* gene from rice *Oryza sativa*. Alignment analysis revealed that OsHAL3 has a high sequence identity to Dfp protein in *Escherichia coli* and AtHAL3a protein in *Arabidopsis thaliana*, which have 4′-phosphopantothenoylcysteine decarboxylase (PPC-DC) activity. *OsHAL3* can complement mutation in the *E. coli dfp* gene encoding PPC-DC, so that the mutant strains with *OsHAL3* can grow on rich media at 42°C and on VB minimal media at 30°C. Complementation tests with point mutations of OsHAL3 suggested that the conserved Cys176 residue of OsHAL3 is a key active-site residue. The mutant OsHAL3 G180A has a partly reduced activity. Related mRNA-level analysis showed that the *OsHAL3* gene is induced by calcium pantothenate in rice.

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Dfp protein in *Escherichia coli* and AtHAL3a protein in *Arabidopsis thaliana* belong to a flavoprotein family that was named HFCD (homo-oligomeric flavin containing Cys decarboxylases) [1, 2]. They have 4′-phosphopantothenoylcysteine decarboxylase (PPC-DC) activity [3, 4]. PPC-DC catalyzes the decarboxylation of (*R*)-4′-phospho-*N*-pantothenoylcysteine (PPC) to 4′-phosphopantetheine (PP), a key step in coenzyme A (CoA) biosynthesis from pantothenate in bacteria and plants [1, 3-6]. PPC-DC is a good target for the development of antimicrobials to treat many diseases [7, 8].

Dfp was originally described as a flavoprotein involved in DNA and pantothenate metabolism. Further study revealed that Dfp protein is a bifunctional enzyme catalyzing the synthesis of PPC and its decarboxylation to PP with ATP [5-7] and CTP [9]. *Escherichia coli dfp-707* mutant can grow on TY (tryptone–yeast) media but required β-alanine or pantothenate for growth on VB (Vogel–Bonner) minimal media at 30°C. The mutant was temperature-sensitive for growth on TY or VB minimal media at 42°C, and it did not grow at 42°C on rich media supplemented with 1 mM pantothenate [10, 11].

The *A. thaliana* flavoprotein AtHAL3a shows sequence homology to the NH_2-terminal domain of Dfp. It is related to salt and osmotic tolerance and to plant growth. Overexpression of the *AtHAL3a* gene improves growth rates and salt and drought tolerance in transgenic *A. thaliana* plants [12]. The X-ray structure of AtHAL3a reveals that the biological activity unit is a trimer [13]. AtHAL3a catalyzes the decarboxylation of PPC to PP *in vitro* [4]. Like Dfp, the conserved Cys175 residue in the substrate recognition clamp of the AtHAL3a protein is essential for this enzymatic activity [1, 5, 14]. The mutant AtHAL3a proteins M145L and C175S showed no PPC-DC activity, whereas the AtHAL3a mutants D177N and G179A had a significantly reduced PPC-DC activity [5]. *NtHAL3a*, *NtHAL3b*, and *NtHAL3c* from *Nicotiana tabacum* complement the temperature-sensitive mutation of the *E. coli dfp* gene [15].

Rice is one of the major grain crops in the world, but the role of the *Oryza sativa OsHAL3* gene is not clear, and rice PPC-DC is little known. In order to reveal whether the OsHAL3 protein has PPC-DC activity, we cloned the

Abbreviations: CoA, coenzyme A; MS, Murashige and Skoog basal salt mixture; PP, 4′-phosphopantetheine; PPC, (*R*)-4′-phospho-*N*-pantothenoylcysteine; PPC-DC, 4′-phosphopantothenoylcysteine decarboxylase; TY, tryptone–yeast medium; VB, Vogel–Bonner medium.

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full-length coding region sequence of the *OsHAL3* gene and investigated its biochemical functions using gene expression analysis and site-directed mutagenesis. Our results provide evidences that OsHAL3 may be a 4′-phosphopantothenoylcysteine decarboxylase.

MATERIALS AND METHODS

Bacterial strains and plant materials. *Escherichia coli* K-12 was kept in our lab. *Escherichia coli* K-12 *dfp-707* mutant strain (the *E. coli* strain BW598) was provided by Dr. Bernard Weiss (Emory University, USA). The *E. coli* strains used were grown in TY medium, VB minimal medium (0.2 g/liter $MgSO_4 \cdot 7H_2O$, 2 g/liter citric acid$\cdot H_2O$, 10 g/liter K_2HPO_4, 3.5 g/liter $NaNH_4HPO_4 \cdot 4H_2O$, 4 g/liter glucose, 1 mg/liter thiamine, and 2 g/liter Norit-treated vitamin-free casamino acids, pH 7.0), or VB minimal medium with 1 mM calcium pantothenate or 1 mM β-alanine (Sigma, USA) [11, 16, 17]. Plasmid-bearing strains were grown in TY medium plus ampicillin (100 μg/ml).

Oryza sativa L. ssp. *japonica* cultivar Nipponbare was grown in a greenhouse at 28°C for 12 h in the dark and 12 h in light. *Arabidopsis thaliana* ecotype Columbia was grown in a greenhouse at 25°C for 8 h in the dark and 16 h in light. Four-week-old seedlings were used for subsequent experiments.

***AtHAL3a* and *OsHAL3* cloning.** The *A. thaliana AtHAL3a* gene and *O. sativa OsHAL3* gene were cloned by reverse transcriptase (RT)-PCR using leaf total RNA as template. Total RNA was prepared from 100 mg of plant tissue of four-week-old seedlings. The samples were homo-genized in 1 ml of Trizol reagent (Invitrogen, USA) in a pre-chilled mortar. For RT-PCR analysis, total RNA was extracted and converted to cDNA using M-MLV reverse transcriptase (Promega, USA) with oligo-$d(T)_{15}$ primers (Promega) according to the manufacturer's instructions.

For RT-PCR, the target genes were amplified from the first strand cDNA template using Taq DNA polymerase and gene-specific primers. For the *AtHAL3* gene, the forward primer was 5′-CTA GGA TCC ATG GAG AAT GGG AAA AGA GAC AGA C-3′ (*Bam*HI site underlined), and the reverse primer was 5′-GTA GAA TTC TTA ACT AGT TCC ACC GGT TTG C-3′ (*Eco*RI site underlined). *OsHAL3* was amplified with a forward primer (5′-CTA GGA TCC ATG ACT ACA TCA GAG TCA GTA C-3′, *Bam*HI site underlined) and a reverse primer (5′-GTA GAA TTC TCA GCT AGA TGG GAG GTT TCT GC-3′, *Eco*RI site underlined). The PCR products were cloned into the pMD18-T vector (Promega) for sequencing. All enzymes used in the experiments were obtained from Promega.

Site-directed mutagenesis of *AtHAL3a* and *OsHAL3*. AtHAL3a and OsHAL3 mutants were constructed by sequential PCR steps [18] using appropriate mutagenesis primers (table). The PCR products were cloned into the pMD18-T vector (Promega) for sequencing.

Plasmid construction and expression in *E. coli*. The PCR-amplified products in pMD18-T were digested with restriction enzymes *Bam*HI/*Eco*RI and directionally cloned into the *E. coli* expression vector pGEX4T-1 (Promega). The constructs were introduced into the *E. coli* K-12 *dfp-707* mutant strain BW598.

Complementation tests with *E. coli dfp-707* strain. Transformants were cultured on TY plates at 30°C for 24 h and 42°C for 12 h for temperature sensitive tests. The pantothenate (or β-alanine) requirement of these strains was tested at 30°C for 48 h on VB minimal agar with or without 1 mM calcium pantothenate or 1 mM β-alanine (Sigma). The bacterial cells were also tested for growth on TY-ampicillin agar to ensure that lack of complementation was not due to plasmid loss [11].

Measurement of the *E. coli* strain growth rates. The bacterial cell densities were estimated from light absorbance at 600 nm as the strains were cultured in TY broths at 140 rpm at 30°C for 8 h. Then they were diluted with water to A_{600} 0.1, and 50 μl of the diluted bacteria were dropped into 5 ml TY broth and cultured in a shaker at 140 rpm at 30 or 42°C. Bacterial cultures were collected at different time points (0, 4, 8, and 12 h) for the measurement of A_{600}.

Analysis of the *OsHAL3* mRNA expression by real-time quantitative PCR. The seeds of rice (*O. sativa* L. ssp. *japonica* cultivar Nipponbare) were de-hulled, surface sterilized and sown on solid agar plates containing 4.3 g/liter Murashige and Skoog (MS) basal salt mixture (Sigma) with 3% (w/v) sucrose and 0.8% (w/v) agar, adjusted to pH 5.8. The MS medium was supplemented with 1 μM calcium pantothenate or 1 μM calcium chloride.

Total RNA was prepared from various plant tissues of two-week-old seedlings. Genomic DNA residuals were eliminated from RNA samples by a DNase (Promega) treatment of 30 min following the manufacturer's instruction.

For real-time quantitative PCR, amplification was performed with OsHAL3-specific primers (forward: 5′-AGA GCA TGG GAC TAC AGC AAA CCA-3′, and reverse: 5′-AAG GCT CAG CCA TTG CAC CAT TAC-3′). To normalize all q-RT-PCR data, the amplification of 18S rRNA was used as an internal control using forward primer (5′-CGG CTA CCA CAT CCA AGG AA-3′) and reverse primer (5′-TGT CAC TAC CTC CCC GTG TCA-3′) [19]. For real-time q-RT-PCR, the cDNA was amplified in the presence of SYBR-Green I intercalating dye (Molecular Probes, USA) using a MyiQ Single Color Real-time PCR Detection System (Bio-Rad, USA). The data obtained were analyzed with MyiQ software (Bio-Rad). Real-time q-RT-PCR experiments were repeated three times independently, and the data were averaged across experiments.

Primers used for site-directed mutagenesis

Gene	Mutation	Primer (mutated bases are underlined)	Orientation	Base pairs
AtHAL3a	M145L	5'-GATTGTTCCACAACAAAGTATTCATAGC-3'	antisense	28
		5'-TACTTTGTTGTGGAACAATCCTTTCA-3'	sense	26
	C175S	5'-TTACCGTAGTCTCCGCTGGCA-3'	antisense	21
		5'-TGCCAGCGGAGACTACGGTAA-3'	sense	21
	D177N	5'-GCTCCATTACCGTAGTTTCCACAGG-3'	antisense	25
		5'-TGGAAACTACGGTAATGGAGCTATG-3'	sense	25
	G179A	5'-GCCATAGCTCCATTAGCGTAGTCTCC-3'	antisense	26
		5'-CTACGCTAATGGAGCTATGGCTGA-3'	sense	24
OsHAL3	M146L	5'-GGGTTGTTCCACAAGAAGGTGTTC-3'	antisense	24
		5'-CACCTTCTTGTGGAACAACCCG-3'	sense	22
	C176S	5'-TAATCACCGCTGGCCAGCC-3'	antisense	19
		5'-GGCCAGCGGTGATTATGGT-3'	sense	19
	D178N	5'-GCACCATTACCATAGTTACCACAGG-3'	antisense	25
		5'-TGGTAACTATGGTAATGGTGCAATGG-3'	sense	26
	G180A	5'-CACCATTAGCATAATCACCACAGG-3'	antisense	24
		5'-CCTGTGGTGATTATGCTAATGGTGC-3'	sense	25

RESULTS AND DISCUSSION

Sequence analyses of OsHAL3, AtHAL3a, and DFP proteins. The *E. coli* Dfp protein NH_2-terminal domain (Met1-Asn190) and the *A. thaliana* AtHAL3a protein have PPC-DC activity [1, 4]. The predicted amino acid sequence of the OsHAL3 protein shows 54.5% identity to the AtHAL3a protein and the N-terminal domain of the Dfp protein and contains four conserved motifs of PPC-DC, including the substrate binding helix, insertion His motif, PXMNXXMW motif, and substrate recognition clamp [4] (Fig. 1).

***OsHAL3* gene complements the *E. coli dfp* mutation.** The *E. coli dfp* gene encodes a PPC-DC [1, 3]. *Escherichia coli* K-12 *dfp-707* mutant cannot grow on VB minimal media without β-alanine or pantothenate at 30°C. It also cannot grow on rich media at 42°C [10, 11]. To investigate whether the OsHAL3 protein has PPC-DC activity, we examined the ability of OsHAL3 to complement the *E. coli* K-12 *dfp-707* mutant. The *OsHAL3* gene was ligated into the *E. coli* expression vector pGEX4T-1 and introduced into the *dfp* mutant (Δdfp + OsHAL3). *Escherichia coli* K12 strain wild-type (Dfp) and *dfp-707* mutant bearing pGEX4T-1-AtHAL3a (Δdfp + AtHAL3a) were positive controls, while *dfp-707* mutant (Δdfp) and *dfp-707* transformant with an empty vector pGEX4T-1 (Δdfp + pGEX4T-1) were negative controls. Complement test showed that the *dfp-707* mutant with *OsHAL3* expression (Δdfp + OsHAL3) grew on TY media at 42°C (Fig. 2a) and also grew on VB minimal media at 30°C without β-alanine or calcium pantothenate (Fig. 2b), indicating that the OsHAL3 protein has a similar function to the AtHAL3a and Dfp proteins.

Analysis of OsHAL3 active-site residues. The conserved Cys158 residue of the substrate recognition clamp of Dfp protein is a key active-site residue for PPC-DC activity *in vitro* [1]. The AtHAL3a mutant proteins with a Cys175 to Ser change (C175S) or a Met145 to Leu (M145L) change in the PPC-DC active site lose PPC-DC activity completely, and the AtHAL3a mutant proteins with an Asp177 to Asn change (D177N) or a Gly179 to Ala change (G179A) showed reduced PPC-DC activity *in vitro* [5, 14].

To elucidate the active-site residues, site-directed mutagenesis of *OsHAL3* was performed. The *E. coli dfp-707* transformants with various mutations of OsHAL3 were cultured on TY plates at 42°C and on VB minimal plates at 30°C. Inability of *E. coli* Δ*dfp* transformants to

grow in the above conditions would indicate that the mutant site of OsHAL3 is essential for its PPC-DC activity. The *dfp-707* strains with corresponding point mutations of AtHAL3a were constructed as controls.

Both *E. coli dfp-707* transformants expressing the mutants OsHAL3 C176S and AtHAL3a C175S could not grow on TY plates at 42°C (Fig. 3a, right), the same as *E. coli dfp-707* (Fig. 2a, right), indicating that neither OsHAL3 C176S nor AtHAL3a C175S mutant protein could complement the *E. coli dfp* phenotype. This result suggests that the conserved Cys176 residue in the substrate recognition clamp of OsHAL3 is essential for its PPC-DC activity, consistent with the *in vitro* PPC-DC enzyme activity test result for AtHAL3a C175S [5, 14]. The mutant AtHAL3a protein M145L could not but the mutant OsHAL3 M146L could complement the *E. coli dfp* lethal phenotype at 42°C (Fig. 3a, right) and grow on VB minimal medium (Fig. 3b, left). This suggests that the Met146 residue of the OsHAL3 protein is not essential for the PPC-DC activity. This result also provided evidence that the OsHAL3 protein may be different from the AtHAL3a in the catalytic mechanism.

Previous *in vitro* analysis demonstrated that AtHAL3a mutant proteins D177N and G179A have reduced PPC-DC activity [5]. *Escherichia coli dfp-707* transformants expressing the mutants AtHAL3a D177N, AtHAL3a G179A, OsHAL3 D178N, and OsHAL3 G180A could grow on TY plates at 42°C and VB minimal plates at 30°C (data not shown). Thus the growth rate of the *E. coli dfp-707* strains expressing the mutants OsHAL3 D178N and OsHAL3 G180A in TY broths were detected at 30 and 42°C. The *E. coli dfp-707* transformant with mutant OsHAL3 G180A protein grew slower than the positive control on TY media at 42°C, while the *E. coli dfp-707* transformant with OsHAL3 D178N grew at similar rates as that of the positive control with OsHAL3 (Fig.

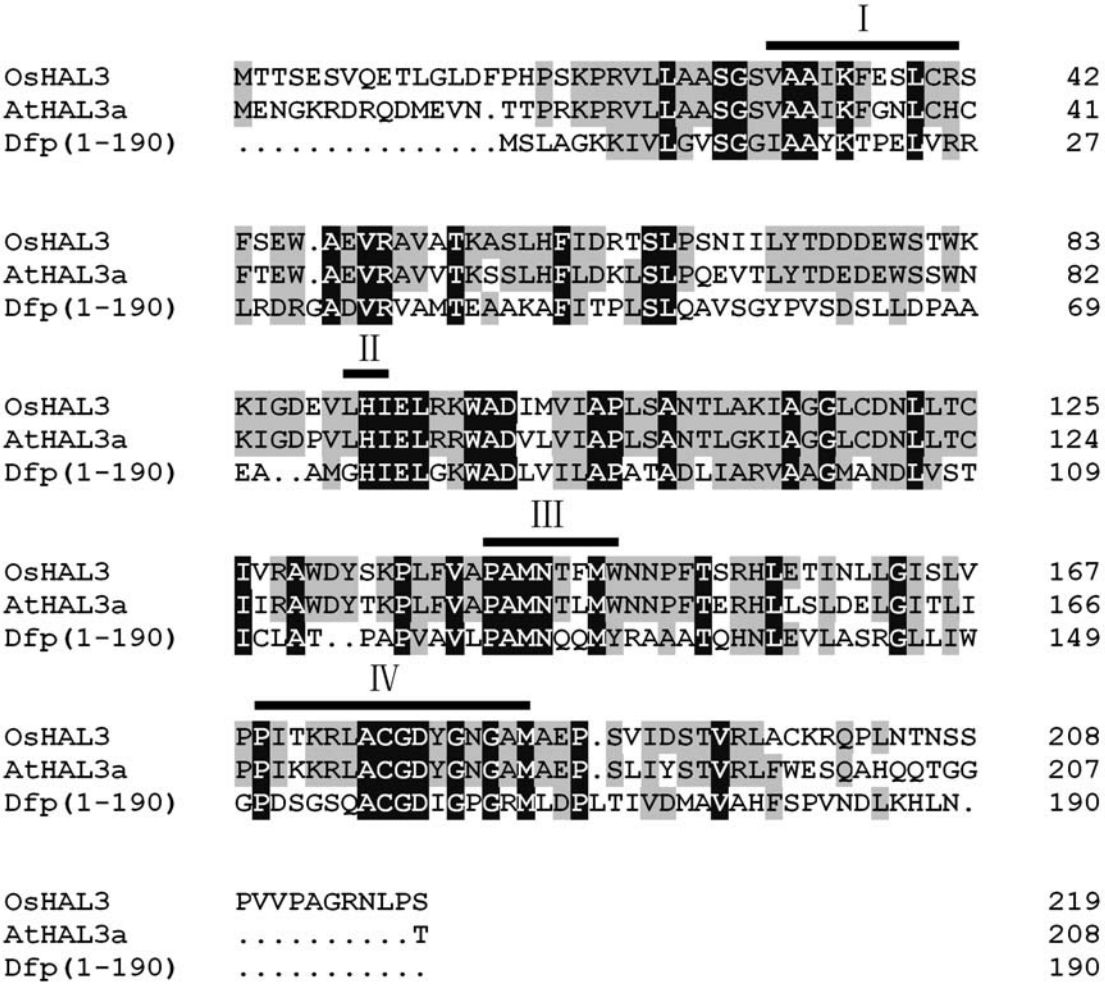

Fig. 1. OsHAL3 alignment with homologous proteins. The predicted amino acid sequence of the *O. sativa* OsHAL3 was compared with homologous representatives from *A. thaliana* and *E. coli*. Identical residues are shown on black background, and conservative substitutions are shaded in gray. Four conserved motifs are labeled as: I, the substrate binding helix; II, insertion His motif; III, PXMNXXMW motif; IV, substrate recognition clamp [4].

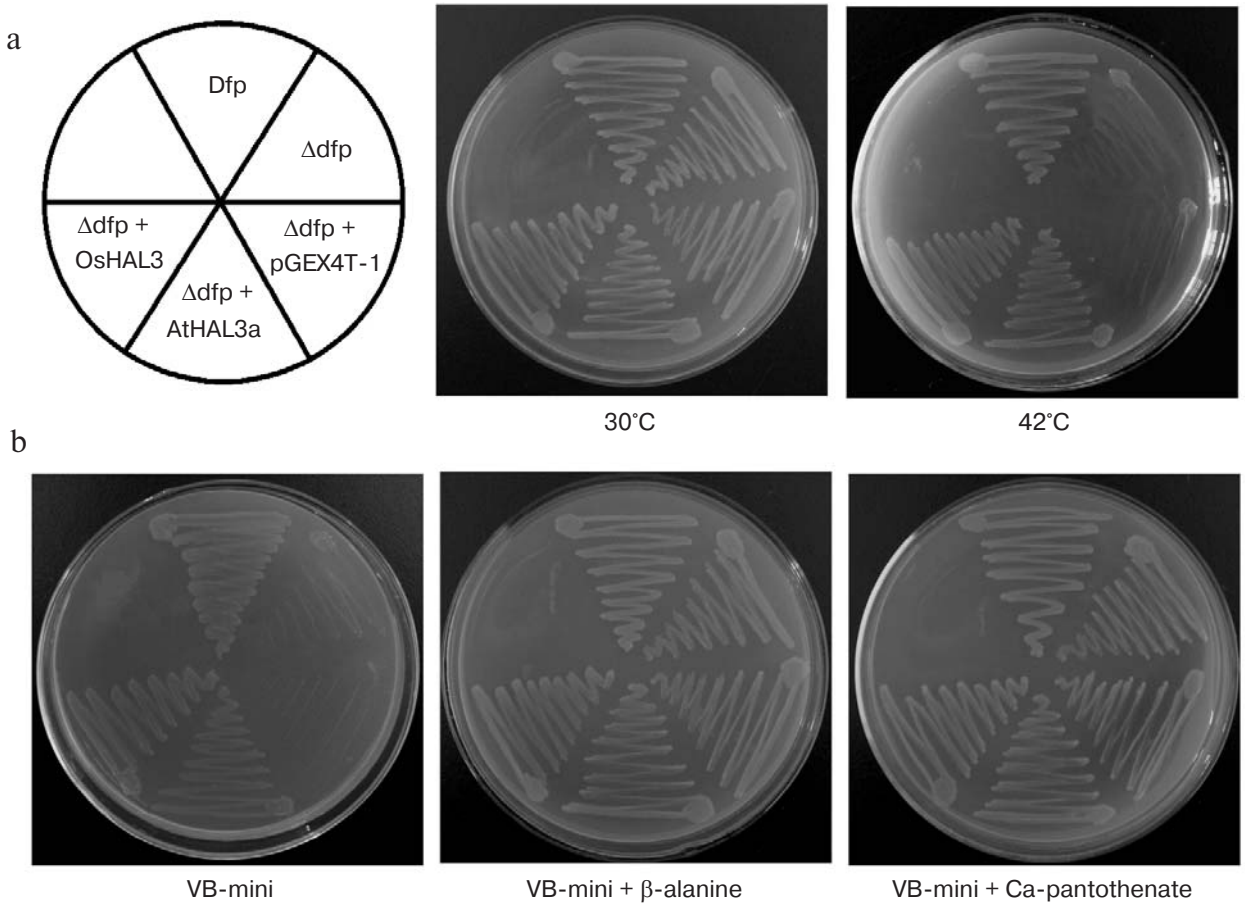

Fig. 2. Complementation analysis of the *OsHAL3* gene with the *E. coli dfp* mutation. *Escherichia coli* K-12 *dfp-707* strain was transformed with an expression vector pGEX4T-1 containing *OsHAL3* gene (Δdfp + OsHAL3). Wild-type *E. coli* K12 strain (Dfp) and *dfp-707* mutant bearing pGEX4T-1-AtHAL3a (Δdfp + AtHAL3a) were positive controls. The *E. coli* mutant *dfp-707* (Δdfp) and *dfp-707* mutant bearing empty vector pGEX4T-1 (Δdfp + pGEX4T-1) were negative controls. a) Transformants were grown on TY plates at 30 and 42°C. b) Transformants were grown on VB minimal medium, VB minimal medium + 1 mM β-alanine, and VB minimal medium + 1 mM calcium pantothenate at 30°C.

4b), suggesting that OsHAL3 G180A has a partly reduced PPC-DC activity and the Asp178 is not important for the activity of OsHAL3.

The *OsHAL3* gene is induced by calcium pantothenate. CoA is synthesized from pantothenate in five steps in plants, archaea, and humans. In the first step, pantothenate is phosphorylated to 4′-phosphopantothenate. Then, PPC is synthesized by the addition of cysteine. In the next step, PPC is decarboxylated to PP by PPC-DC. Then, PP is converted to CoA [3-6, 20].

The *dfp-707* mutant required a minimum of 0.45 μM pantothenate for growth on solid minimal media [11], so it was predicted that the *dfp* gene probably could be induced by pantothenate, and other genes encoding PPC-DC may also be induced by pantothenate. In order to examine whether the *OsHAL3* gene plays a role in the biosynthesis in CoA from pantothenate, we analyzed the gene expression with treatment of calcium pantothenate. Calcium chloride was used as a control to avoid the interference of calcium. We used real-time RT-PCR to analyze the expression of *OsHAL3* in 2-week-old seedlings on treatment with calcium pantothenate. The results showed that *OsHAL3* mRNA is induced by calcium pantothenate, but not by calcium chloride (Fig. 5) and provided evidence that OsHAL3 protein plays a functional role in the CoA biosynthetic pathway.

In conclusion, we demonstrated that the OsHAL3 protein is likely to be a rice 4′-phosphopantothenoylcysteine decarboxylase, and the conserved Cys176 residue is the key active site residue. Our findings shed new light on our understanding of the possible catalytic mechanism of PPC-DC *in vitro*. Further investigation is needed to determine its biological roles *in vivo*. For example, overexpression of *OsHAL3* in rice, site-directed mutagenesis on more active-site residues, and determination of the three-dimensional structure of the OsHAL3 protein will help to gain more details about the function of this protein and determine its difference from AtHAL3a protein. Such investigations would also enable us to elucidate the mechanisms underlying CoA biosynthesis in plants.

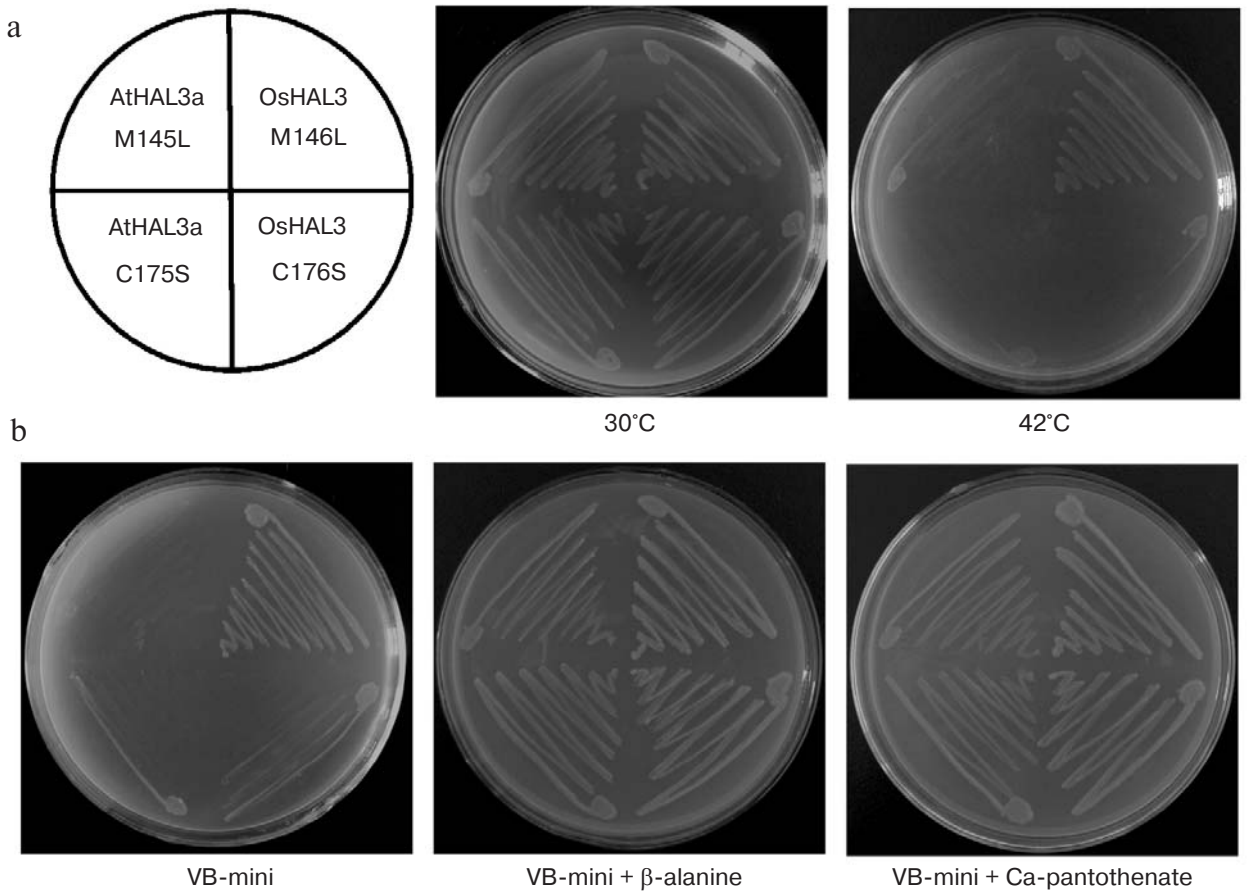

Fig. 3. Complementation analysis of mutant *OsHAL3* genes. The *E. coli dfp-707* mutant strains with active site mutant genes of *OsHAL3* and *AtHAL3a* were tested. a) Transformants were grown on TY plates at 30 and 42°C. b) Transformants were grown on VB minimal medium, VB-mini + 1 mM β-alanine, or VB-mini + 1 mM calcium pantothenate plates at 30°C.

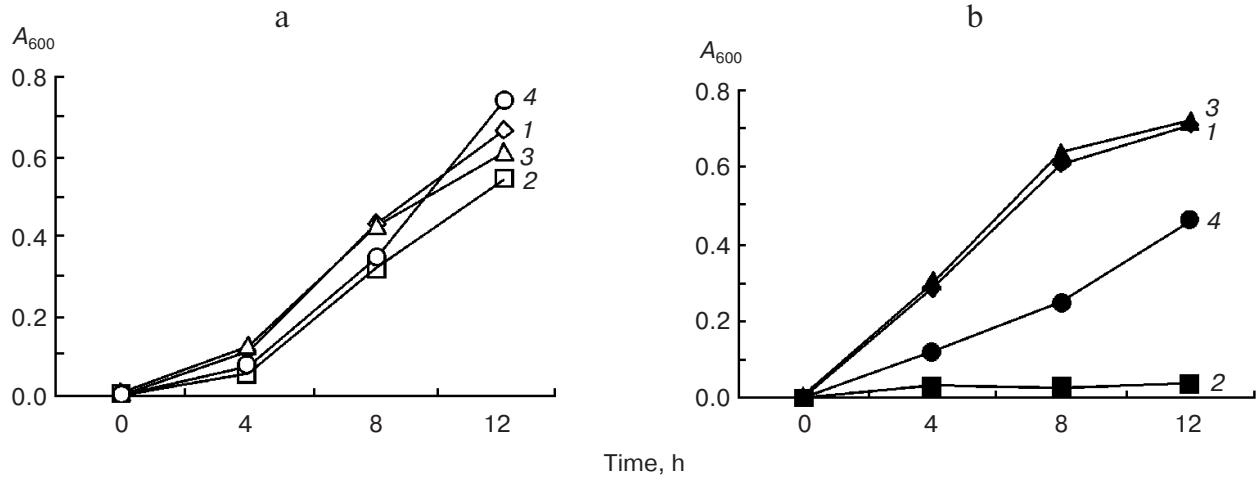

Fig. 4. Growth rates of the *E. coli dfp-707* strains bearing various mutant plasmids. Cells of *E. coli dfp-707* mutant strains expressing mutant *OsHAL3* genes were cultured in TY broths and shaken at 140 rpm at (a) 30°C and (b) 42°C for detection of A_{600} at 0, 4, 8, and 12 h, respectively: *1*) *E. coli dfp-707* mutant bearing pGEX4T-1-OsHAL3 as positive control; *2*) *E. coli dfp-707* mutant bearing pGEX4T-1 as negative control; *3*) *dfp-707* mutant bearing pGEX4T-1-OsHAL3 D178N; *4*) *dfp-707* mutant bearing pGEX4T-1-OsHAL3 G180A. Each data point represents the average of three independent determinations.

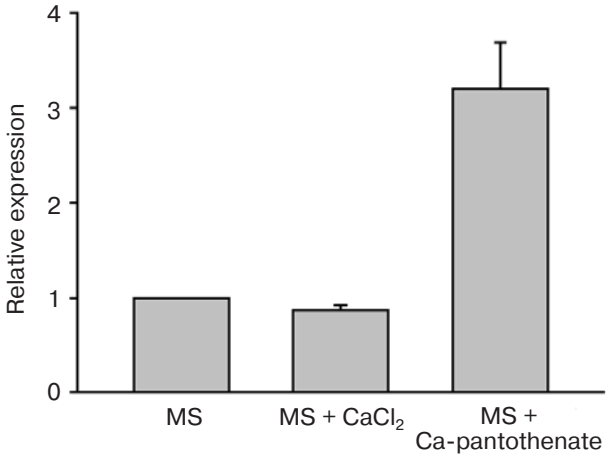

Fig. 5. *OsHAL3* relative expression in *O. sativa* with calcium pantothenate treatment. Total RNAs were extracted from 2-week-old seedlings in the real-time PCR. The seedlings were treated on MS, MS with 1 μM calcium chloride, and MS plus 1 μM calcium pantothenate. Error bars represent SD.

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