

## Deletion mutation analysis on C-terminal domain of plant vacuolar H<sup>+</sup>-pyrophosphatase

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### Abstract

Vacuolar H<sup>+</sup>-translocating inorganic pyrophosphatase (V-PPase; EC 3.6.1.1) is a homodimeric proton-translocase; it contains a single type of polypeptide of approximately 81 kDa. A line of evidence demonstrated that the carboxyl terminus of V-PPase is relatively conserved in various plant V-PPases and presumably locates in the vicinity of the catalytic site. In this study, we attempt to identify the roles of the C-terminus of V-PPase by generating a series of C-terminal deletion mutants over-expressed in *Saccharomyces cerevisiae*, and determining their enzymatic and proton translocating reactions. Our results showed that the deletion mutation at last 5 amino acids in the C-terminus ( $\Delta C5$ ) induced a dramatic decline in enzymatic activity, proton translocation, and coupling efficiency of V-PPase; but the mutant lacking last 10 amino acids ( $\Delta C10$ ) retained about 60–70% of the enzymatic activity of wild-type. Truncation of the C-terminus by more than 10 amino acids completely abolished the enzymatic activity and proton translocation of V-PPase. Furthermore, the  $\Delta C10$  mutant displayed a shift in  $T_{1/2}$  (pretreatment temperature at which half enzymatic activity is observed) but not the optimal pH for PP<sub>i</sub> hydrolytic activity. The deletion of the C-terminus substantially modified apparent K<sup>+</sup> binding constant, but exert no significant changes in the Na<sup>+</sup>-, F<sup>-</sup>-, and Ca<sup>2+</sup>-inhibition of the enzymatic activity of V-PPase. Taken together, we speculate that the C-terminus of V-PPase may play a crucial role in sustaining enzymatic activity and is likely involved in the K<sup>+</sup>-regulation of the enzyme in an indirect manner.

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**Keywords:** Proton translocation; Proton translocase; Tonoplast; Vacuole; Vacuolar H<sup>+</sup>-pyrophosphatase; Inorganic pyrophosphate; Heterologous expression; C-terminal truncation; Deletion mutagenesis; K<sup>+</sup>-binding

Plant vacuolar proton-translocating inorganic pyrophosphatase (V-PPase, EC 3.6.1.1)<sup>1</sup> generates an H<sup>+</sup> electrochemical gradient across tonoplast membrane for the transport of solutes at the expense of hydrolyzing pyrophosphate [1–3]. Vacuolar H<sup>+</sup>-PPase is a homodimeric protein consisting of a single kind of polypeptide with a molecular mass of about 75–81 kDa [1,4,5]. The cDNAs of V-PPase have been cloned from various higher plants,

several bacteria, and some protists, and showed high similarity (86–91% identity) in their amino acid sequences [1,6,7]. The enzymatic activity of V-PPase could be stimulated by relative high concentration of K<sup>+</sup>, but inhibited by F<sup>-</sup>, Na<sup>+</sup>, Ca<sup>2+</sup>, and excess PP<sub>i</sub>, respectively [1,8–11]. In addition, several K<sup>+</sup>-independent isoforms of V-PPases from *Arabidopsis*, *Rhodospirillum rubrum*, and *Pyrobaculum aerophilum* have been recently found [6,12–14]. This novel subclass of K<sup>+</sup>-independent V-PPase is relatively resistant to Na<sup>+</sup> inhibition [12,13]. Furthermore, a line of evidence indicated that both subtypes of PP<sub>i</sub>-supported proton-translocases possess presumably similar structural features, but with relatively low identity in amino acid sequence [6,12,14].

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<sup>1</sup> Abbreviations used: DDT, dithiothreitol; EDTA, N,N,N',N'-ethylenediamine tetraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethyl-ether)N,N,N',N'-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; V-PPase, vacuolar H<sup>+</sup>-pyrophosphatase.

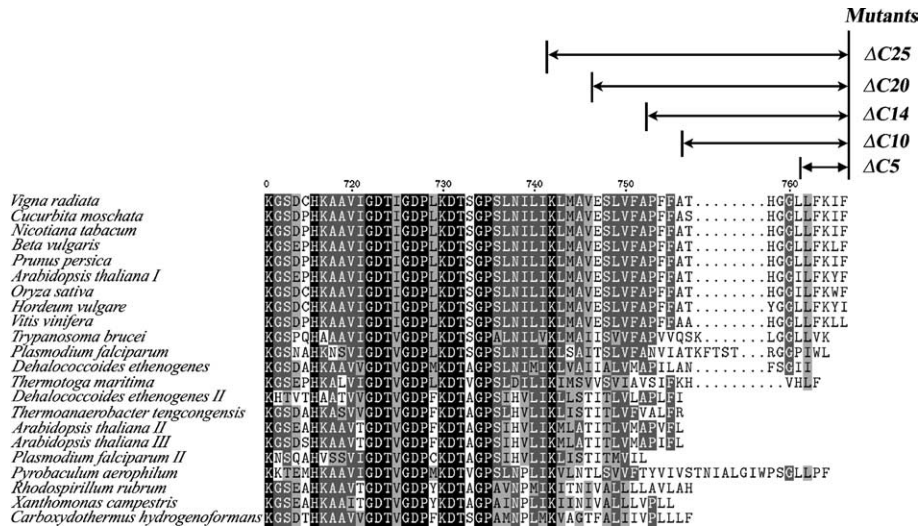


Fig. 1. Sequence alignment of C-terminal regions from various vacuolar H<sup>+</sup>-PPases of higher plants and bacteria. Amino acid sequences of C-terminal regions in vacuolar H<sup>+</sup>-PPases from various sources were aligned using Clustal X program [33]. Residue numbers on the top of alignments indicate the residue location of mung bean vacuolar H<sup>+</sup>-PPase. The GenBank Accession Nos. for V-PPases of each species: *Vigna radiata*, P21616; *Cucurbita moschata*, BAA33149; *Nicotiana tabacum*, S61423; *Beta vulgaris*, AAA61609; *Prunus persica*, AAL11507; *Arabidopsis thaliana*, A38230; *Oryza sativa*, BAA08232; *Hordeum vulgare*, BAA02717; *Vitis vinifera*, AAF69010; *Trypanosoma brucei*, AAK95376; *Plasmodium falciparum*, AAD17215; *Dehalococcoides ethenogenes*, TIGR\_243164|Ucontig6871; *Thermotoga maritima*, D72409; *Dehalococcoides ethenogenes II*, TIGR\_243164|Ucontig6871; *Thermoanaerobacter tengcongensis*, AAM23580; *Arabidopsis thaliana II*, AAF31163; *Arabidopsis thaliana III*, AAG09080; *Plasmodium falciparum II*, AAG21366; *Pyrobaculum aerophilum*, AAF01029; *Rhodospirillum rubrum*, AAC38615; *Xanthomonas campestris*, AAM42582; *Carboxydotherrmus hydrogenoformans*, TIGR\_246194U|contig2356. “TIGR” denotes sequence obtained from the unfinished microbial genome databases of The Institute for Genomic Research.

The essential regions of V-PPases for enzymatic function, proton translocation, and binding sites of ions, such as K<sup>+</sup>, F<sup>-</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup> have not yet been determined. However, several studies using chemical modification and site-directed mutagenesis have identified many important residues and fragments involved in both enzymatic and proton translocating reactions. For instance, modification of purified V-PPase with arginine- and tyrosine-specific reagents resulted in a marked decline in enzymatic and H<sup>+</sup>-translocating activities, indicating that these residues are essential and might locate in/or near the catalytic domain of the enzyme [15,16]. By mutational analysis, several charged residues have also been shown to be crucial for the enzymatic activity [1,17,18]. Further parallel mutational and biochemical analyses identified a histidine and several carboxylic residues rendering inhibition of V-PPase by diethylpyrocarbonate and *N,N'*-dicyclohexylcarbodiimide, the histidyl and the carboxyl group specific modifiers, respectively [19–22]. Moreover, mutational studies demonstrated that two consensus acidic motifs of DX<sub>3</sub>DX<sub>3</sub>D might be the candidates for substrate binding and the energy conversion from PP<sub>i</sub> hydrolysis to H<sup>+</sup> transport [17]. In addition, phylogenetic analysis together with site-directed mutagenesis revealed a possible contribution in K<sup>+</sup> binding at A460 of H<sup>+</sup>-PPase from *Carboxydotherrmus hydrogenoformans* [23]. Substitution of A460 by the lysine residue could convert H<sup>+</sup>-PPase of *C. hydrogenoformans* from K<sup>+</sup>-sensitive to K<sup>+</sup>-insensitive forms [23].

Alignment analysis of amino acid sequence demonstrated relatively higher degree in conservation of the C-termi-

nal domain (90%) than that (<40%) of the N-terminal portion among various V-PPases (Fig. 1). Specific antibody to a portion of the carboxyl-terminus of V-PPase could suppress its hydrolytic and proton pumping activities, suggesting its potential location in the vicinity of the catalytic site [24]. It is thus conceivable that C-terminus is presumably more important for functional V-PPase. However, C-terminus of V-PPases from many protists and some prokaryotes lacks of approximately last 10 amino acid residues of plant V-PPase [2]. In addition, the predicted localization of the C-terminus of plant V-PPase and that of *Streptomyces coelicolor* determined experimentally are also different [6,25]. In this study, we thus attempted to identify the roles of the C-terminus of plant V-PPase by the C-terminal deletion method. A series of C-terminus truncated mutants were constructed, over-expressed in *Saccharomyces cerevisiae*, and their enzymatic activities and proton translocation determine. Our results indicate the deletion of the C-terminus induces a dramatic decline of V-PPase in enzymatic activity, proton translocation, and coupling efficiency. In addition, removal of the C-terminus augmented heat vulnerability and also substantially increased the apparent K<sup>+</sup>-binding constant of V-PPase.

**Materials and methods**

*Heterologous expression of mung bean V-PPase in yeast*

A *HindIII*–*XbaI* fragment of the cDNA encoding mung bean V-PPase (Accession No. P21616) was inserted into the

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