

A 55-kDa Calcium Dependent Protein Kinase Phosphorylated Thr Residues from the Auto-Regulatory Domain of Plasma Membrane H⁺-ATPase in Rice

Tetsuya OOKURA^{1*}, Setsuko KOMATSU², Yukio KAWAMURA³ and Kunihiro KASAMO⁴

^{1,3} Food Function Division, National Food Research Institute (Tsukuba, Ibaraki 305–8642, Japan)

^{2,4} Department of Molecular Genetics, National Institute of Agrobiological Sciences (Tsukuba, Ibaraki 305–8602, Japan)

⁴ Resource Institute of Botany, Okayama University (Kurashiki, Okayama 710–0046, Japan)

Abstract

Plasma membrane H⁺-ATPases play important roles in plant growth by keeping a proton gradient across the membrane. Although cold or pathogen stress affects the activity of the H⁺-ATPase and plant growth, its regulatory mechanisms are not fully elucidated. A rice 55-kDa calcium-dependent protein kinase was activated on cold treatment and pathogen infection. Here we showed that the kinase phosphorylated two Thr residues in the auto-regulatory domain of the H⁺-ATPase *in vitro*. Phosphorylation of these Thr residues might contribute to the ATPase activity regulation during cold or pathogen stress.

Discipline: Biotechnology

Additional key words: ATPase activity, cold stress

Introduction

Plasma membrane H⁺-translocating ATPases (H⁺-ATPases) from plants are electrogenic proton pumps which regulate intracellular pH and nutrient uptake. The control of H⁺-ATPase activity is critical to plant growth. Proteolytic digestion of the H⁺-ATPases clarified that the trypsin cleavable C-terminal region (auto-regulatory domain; ~100 amino acids) regulated ATPase activity. N-terminal side of the auto-regulatory domain (Region I) and extreme C-terminal part of the domain (Region II) have been reported to have different effects on the ATPase activity. Extensive analysis on Region II revealed that phosphorylation of the penultimate Thr residue in the C-terminal end resulted in binding of 14-3-3 proteins towards the Region II, which led to constitutive activation of the ATPase activity^{4,5}. Although Region I peptide suppressed the enhancement of the ATPase activity by trypsin cleavage¹⁹, the regulatory mechanism remains to be elucidated.

It has been suggested that membrane-bound forms

of Ca²⁺-dependent protein kinases (CDPKs) phosphorylate the H⁺-ATPases. Evidence for the calcium-dependent phosphorylation of the H⁺-ATPases has been accumulated in studies on the beet root¹³, fava bean guard cells²⁰ and oat root²¹. In rice, a 55-kDa CDPK has been reported to function near the plasma membrane on cold stress treatment¹² and pathogen infection¹¹. The substrates for the 55-kDa CDPK are, however, largely unknown. The mechanism for growth retardation associated with cold or pathogen stress remains to be elucidated.

Here we report that the antibody against the Region I peptide augmented the ATPase activity *in vitro* and the 55-kDa CDPK phosphorylated two Thr residues in the peptides from the Region I in a calcium dependent manner. These results might clarify the regulatory mechanism of the ATPases activity by CDPK and lead to understanding how cold or pathogen stress affects rice growth through regulation of the electrogenic H⁺-ATPase pump.

Present address:

³ Department of Food and Nutrition, Kinki University (Nakamachi, Nara 631–8505, Japan)

*Corresponding author: fax +81–29–838–7319; e-mail ookura@nfri.affrc.go.jp

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Materials and methods

1. Chemicals

Fmoc-amino acids (Fmoc, fluorene-9-ylmethoxy-carbonyl) were purchased from Nova Biochem (Tokyo, Japan). [γ - 32 P]ATP was obtained from Amersham Biosciences (Piscataway, NJ, USA).

2. Peptide preparation and antibody production

Auto-regulatory domain peptides and mock peptides were synthesized on HMP (Hydroxy-methylphenoxymethyl) resin or MAP (Multiple antigen peptide) resin with a peptide synthesizer (430A, Applied Biosystems, Foster City, CA, USA) using Fmoc-amino acids. The protected residues were deblocked in trifluoroacetic acid (TFA) in the presence of thioanisole and two additional scavengers, *m*-cresol and ethanedithiol. Each peptide was purified through reversed phase HPLC on an ODS column with a linear gradient of 0% to 60% acetonitrile in 0.1% TFA for 60 min or gel chromatography using Sephadex G-50 (Amersham Biosciences) for MAP resins as described previously¹⁷. The amino acid sequence of peptides was determined with a gas-phase protein sequencer (473A, Applied Biosystems). Peptide quantities were determined by using an amino acid analyzer (Hitachi 5500, Tokyo, Japan). The purified MAP peptide, containing the amino acid sequence (RIAFTRKKDFGTQENQLKWATAQRRTI; AR-1), was used to raise an antibody in mice with Freund's adjuvant using a standard protocol. Mouse sera were purified by ammonium sulfate precipitation. Antibody concentration was determined by protein assay kit (Biorad, Hercules, CA, USA) using bovine serum albumin as a standard.

3. ATPase activity measurement with trypsin or antibody treatment

ATPase activity was measured as described previously⁸. Antibody or trypsin and 27.5 μ g solubilized ATPase were mixed and preincubated at 38°C for 30 min in 30 μ L ATPase reaction mixture containing 3 mM Tris-ATP, 3 mM MgSO₄, 50 mM KCl, 2 mM gramicidin D, 1 mM sodium azide and 1 mM ammonium molybdate in 30 mM MES/Tris (pH 6.5). In the case of trypsin treatment, protease digestion was terminated by addition of trypsin inhibitor (50 μ g eq.) prior to the addition of ATPase reaction mixture (0.5 mL). Inorganic phosphate production by ATPase was measured in the presence of 0.02% aroclorin during incubation at 38°C for 30 min.

4. A 55-kDa calcium dependent protein kinase preparation from rice

The crude extract from rice leaves was prepared

according to the method of Kanematsu⁶. A 55-kDa CDPK was partially purified through DEAE MemSep 1010 column (Millipore, Bedford, MA, USA), Sephacryl S-200 (1,000 \times 21 mm, Amersham Biosciences) and Threonine-Sepharose column (40 \times 26 mm, Amersham Biosciences) as previously reported⁷. This fraction was used as a 55-kDa CDPK fraction.

5. *In vitro* phosphorylation of Region I peptide by calcium dependent protein kinases

The *in vitro* phosphorylation assay was carried out in a reaction mixture (40 μ L) containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 200 μ M CaCl₂, 34.2 μ M [γ - 32 P]ATP (0.44 TBq/mmol), 5 μ L protein kinase and 2 μ g histone III-S (Sigma, St. Louis, MO, USA) or 1 nmole peptide. The basal level of substrate phosphorylation was measured in the presence of 4 mM EGTA instead of CaCl₂. The reaction mixture was incubated for 15 min at 30°C and terminated by addition of SDS-PAGE sample buffer. After *in vitro* phosphorylation, the proteins were separated by low bis SDS-PAGE and stained with Coomassie brilliant blue (CBB) as previously described⁷. The gel was destained, dried and exposed to autoradiography on an X-ray film (Kodak, Rochester, NY) at -80°C for 2 days.

6. Assay of protein kinase activity

Protein kinase activity was assayed in a reaction mixture (100 μ L) containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.4 μ g/mL histone III-S or 1 nmole peptide, 200 μ M CaCl₂, 34.2 mM [γ - 32 P]ATP (110 TBq/mmol) and 5 μ L of rice protein kinase fraction. The basal level of substrate phosphorylation was measured in the presence of 4 mM EGTA instead of CaCl₂. The reaction mixture was incubated for 15 min at 30°C and terminated by cooling to 0°C. Then 75 μ L of reaction mixture was applied onto a P-81 filter (Whatman, Maidstone, UK). The filters were washed with water followed by ethanol, then dried. Radioactivity was determined by a Cherenkov counting.

Results and discussion

1. ATPase activity modulation by trypsin and antibody treatment

The auto-regulatory domain cleavage by trypsin in oat root and sugar beet leaf H⁺-ATPase resulted in increasing ATPase activity^{18,19}. Fig. 1 shows the possible modes of H⁺-ATPase regulation by proteolytic digestion, phosphorylation and antibody treatment on the auto-regulatory domain. Treatment of the rice H⁺-ATPase with 5 μ g trypsin gave maximal augmentation of the ATPase

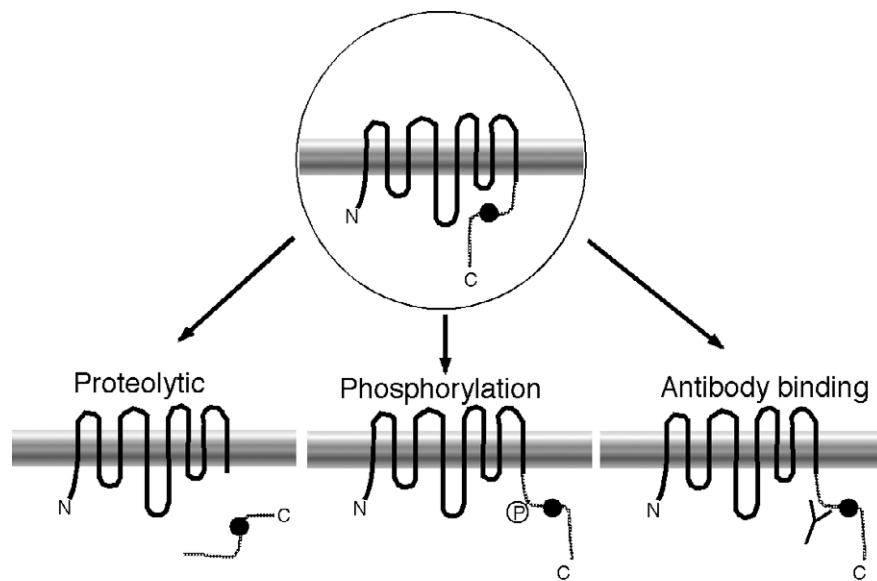


Fig. 1. Possible modes of the H⁺-ATPase activity regulation by the C-terminal auto-regulatory domain

The auto-regulatory domain in the C-terminal (hatched lines) was believed to interact with another domain to inhibit ATPase activity. Proteolytic digestion of the domain and phosphorylation in the extreme C-terminal Thr residues resulted in increasing H⁺-ATPase activity.

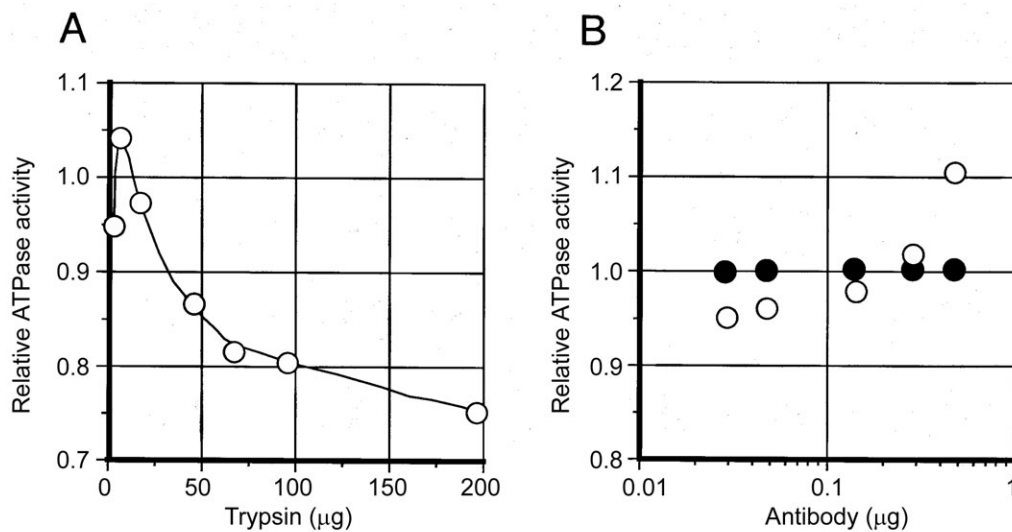


Fig. 2. ATPase activity modulation by trypsin and anti-AR-1 peptide antibody treatment

27.5 µg of solubilized ATPase in the ATPase reaction mixture was treated with either trypsin (A) or anti-AR-1 antibody (B). In the case of trypsin treatment (A), maximal augmentation was observed at 5 µg trypsin treatment. On antibody treatment (B), open circles represented the activity change of the ATPase by the addition of the anti-AR-1 peptide antibody. Filled circles represented the activity for addition of equivalent control mouse IgG.

activity (Fig. 2A). Activity decrease on treatment with over 50 µg trypsin might be due to other domain proteolytic cleavage. Addition of anti-AR-1 peptide (RIAFTRKKDFGTQENQLKWATAQRTI) antibody (0.5 µg; open circle) led to augmented ATPase activity (110%), while control mouse IgG addition (filled circles)

did not have an effect on the ATPase activity (Fig. 2B). These results indicated that the AR-1 sequence had an inhibitory effect on ATPase activity. This fact was consistent with the report that external addition of the auto-regulatory domain peptide (AFTMKKDYGKKEEREAQ WALAQRTLHGLQ) suppressed the ATPase activity of

the trypsin-activated ATPase¹⁹. These results strongly suggest that the AR-1 peptide region in the auto-regulatory domain plays an inhibitory role in regulating ATPase activity.

Calcium dependent phosphorylation and de-phosphorylation of the H⁺-ATPases have been reported¹. Considering that the auto-regulatory domain was easily cleaved by trypsin and was bound by the anti-AR-1 antibody, we assumed that this domain was likely to be accessible to calcium dependent protein kinases (CDPKs). The rice 55-kDa CDPK was localized at the membrane fraction on cold treatment¹² and pathogen infection¹¹. Thus we next analyzed whether the AR-1 peptide was phosphorylated by the 55-kDa CDPK.

2. AR-1 phosphorylation by CDPK *in vitro*

We analyzed whether the 55-kDa CDPK phosphorylated the AR-1 related peptides, which were based on the peptide sequence in the Region I of the auto-regulatory domain of the rice H⁺-ATPase (OSA2)¹⁶, with two methods. First we analyzed phosphorylation of the AR-1 using SDS-PAGE and autoradiography. Lane 1 in Fig. 3 showed the CBB-staining of AR-1 (indicated by a closed rectangular). Lanes 4 and 5 showed that the AR-1 was phosphorylated in the presence of 200 μ M CaCl₂ (lane 5), but not in the absence of CaCl₂ (lane 4). AR-1 had, however, no phosphorylating activity by itself (lane 3). These results indicated that the 55-kDa CDPK phosphorylated the AR-1 in a calcium dependent manner. Lane 5 contained a faint band (indicated by an arrow) above the main band. The Chou and Fasman method predicted that the AR-1 was able to form an α -helix structure². The faint band might be AR-1 aggregates through helix to helix interactions.

Next, we determined which Thr residues in the AR-1 sequence were phosphorylated by the 55-kDa CDPK.

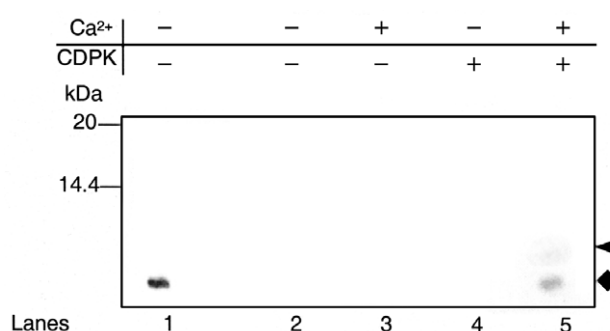


Fig. 3. AR-1 peptide (RIAFTRKKDFGTQENQLKWATAQRTI) was phosphorylated in a calcium-dependent manner

Lane 1: CBB staining of the AR-1. Lanes 2 to 5: Autoradiogram of the AR-1 peptide. The AR-1 peptide was incubated with CaCl₂ (lanes 3 and 5) or without CaCl₂ (lanes 2 and 4) in the presence of protein kinase fraction (lanes 4 and 5) or the absence of protein kinase fraction (lanes 2 and 3). A filled rectangular showed the AR-1 peptide location. An arrow showed a supposed AR-1 aggregate in lanes 1 and 5.

Five kinds of peptides (AR-1 to AR-5, Table 1) were subjected to the *in vitro* phosphorylation assay. Table 1 showed that AR-1, AR-3 and AR-4 were phosphorylated in a calcium dependent manner. All three peptides contained Thr (869) and/or Thr (885). AR-2 (Thr 875) and AR-5 (Thr 889) were not phosphorylated by the CDPK. These data indicated that Thr 869 (a conserved Thr residue among plant H⁺-ATPases) and Thr 885 (a unique Thr in OSA2) were phosphorylated by the 55-kDa CDPK in a calcium dependent manner *in vitro*.

3. ATPase activity regulation by CDPK phosphorylation

We discussed about the results of ATPase phospho-

Table 1. CDPK phosphorylated two Thr residues of H⁺-ATPase auto-regulatory peptides

Substrate	Protein kinase activity (cpm)		
	865 *	* 890	
AR-1	RIAFTRKKDFGTQENQLKWATAQRTI		82
AR-2	QRIAFVRKKDFGTQE		1
AR-3	QRIAFTRKKDFGVQE		52
AR-4		NQLKWATAQRVI	29
AR-5		NQLKWAVAQRTI	1
Control ; histone III-S			379

CDPK activity was assayed with each substrate in the presence of 100 μ M CaCl₂. The basal level of substrate phosphorylation was measured in the presence of 4 mM EGTA instead of CaCl₂. All assays were means of duplicate determinations from independent experiments. The AR-1 peptide (865–890) sequence was deduced from the cDNA data.

* : Conserved Thr residues among plant plasma membrane H⁺-ATPases.

rylation by the 55-kDa CDPK from two points of view, one from the roles of the CDPK and another from possible ATPase regulation.

Evidence for calcium dependent phosphorylation of ATPase has been accumulated^{1,3,13,20,21}. Lino et al. showed that calcium dependent phosphorylation of H⁺-ATPase in beet root led to inhibition of ATPase activity. Kinoshita et al. reported calcium dependent inhibition of ATPase activity in guard cells of fava bean¹⁰. CDPK phosphorylation residues in other species of the H⁺-ATPase have not been, however, identified yet. In this report, we showed that the 55-kDa CDPK phosphorylated the two Thr residues in the Region I of the rice H⁺-ATPase. The rice 55-kDa CDPK was activated on low temperature treatment¹² and pathogen infection¹¹. Thr (869) and Thr (885) phosphorylation might couple to H⁺-ATPase activity modulation on cold stress and pathogen infection. Interestingly, it is reported by Martin et al. that 56-kDa CDPK and OsCPK2, which are membrane-bound, were activated in response to low temperature^{14,15}. These facts raised the possibility that the 55-kDa CDPK in this study might be an OsCPK2-like kinase.

In a reconstituted system, phospholipids have been shown to modulate the H⁺-ATPase activity and H⁺-pumping. Lysophosphatidylcholine, which had a positive charge at its hydrophilic region, increased ATPase activity⁹. These facts suggest that ionic interaction between ATPase and phospholipids appears to play a pivotal role for ATPase activity. Deletion of Region I plus Region II resulted in loss of phospholipid modulation of ATPase activity. The Thr (869) residue is adjacent to a positively charged cluster (RKK). Phosphorylation of the Thr (869) residue can neutralize positive charges around the cluster. This charge change may consequently alter the interaction of the Region I with phospholipids leading to modulation of ATPase activity. *In vivo* phosphorylation analysis with specific antibodies against phosphothreonine around Thr (869) and Thr (885) will clarify which residues are phosphorylated or dephosphorylated upon ATPase activity regulation. If the phosphorylation on Thr residues is detected *in vivo* on cold or pathogen infection, mutations on the Thr residues in the H⁺-ATPase might be a target for breeding cold or pathogen tolerant rice.

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