Regulation of the structure and catalytic properties of plasma membrane H^+-ATPase involved in adaptation of two reed ecotypes to their different habitats

K.-M. CHEN*,***[1], H.-J. GONG**, S.-M. WANG**, W.-J. ZHENG* and C.-L. ZHANG**

*Institute of Botany, Research Center of Molecular & Developmental Biology, Chinese Academy of Sciences, Beijing, 100093, P.R. China**
*School of Life Sciences, Lanzhou University, Lanzhou, 730000, P.R. China***
*College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, 310029, P.R. China****

Abstract

The properties and kinetics of ATP and *p*-nitrophenyl phosphate (PNPP) hydrolysis activities of plasma membrane H^+-ATPase from the two reed ecotypes, swamp reed (SR) and dune reed (DR), were investigated. The pH optimum of the plasma membrane H^+-ATPase in both reed ecotypes was similar but the sensitivity of the enzyme to the reaction medium pH seemed to be higher in DR than that in SR. Compared to SR, the DR exhibited a higher V_{max} value for ATP hydrolysis whereas the K_m value was almost similar in both reed ecotypes. The PNPP hydrolysis of the plasma membrane H^+-ATPase was also studied in both reed ecotypes at increasing PNPP concentrations. K_m and V_{max} for PNPP hydrolysis showed great differences in the two reed ecotypes and in DR the K_m and V_{max} values were 2- and 10-fold, respectively, higher than those in SR. The ATP hydrolysis activity of the plasma membrane was markedly inhibited by hydroxylamine in both reed ecotypes, and the percentage inhibition of ATP hydrolysis rate seemed higher in DR than that in SR. In addition, the structure or property of the C-terminal end of the plasma membrane H^+-ATPase were also different in the two reed ecotypes. These data suggest that different isoforms of the plasma membrane H^+-ATPase might be developed and involved in the adaptation of the plant to the long-term drought-prone habitat.

Additional key words: ATP and PNPP hydrolysis, *Phragmites communis*.

Introduction

The proton-pumping ATPase (H^+-ATPase) of the plant plasma membrane, can generate the proton motive force across the plasma membrane that plays a major role in activating most of the ion and metabolite transport (Serrano 1989, Morsomme and Boutry 2000). This enzyme belongs to the family of P-type ATPases because it has a catalytic subunit of *ca.* 100 kDa that forms a phosphorylated intermediate sensitive to vanadate (Serrano 1989). It plays a central role in the growth and development of plants and is subjected to modulation by many environmental factors, including toxin, light, injury, mineral nutrients and other biotic and abiotic constrains (Michelet and Boutry 1995, Morsomme and Boutry 2000). The plasma membrane H^+-ATPase has ten transmembrane helixes, which consist of 3 functional domains: the phosphatase domain, the transduction domain and the kinase domain. The phosphatase domain catalyses the dephosphorylation of the phosphorylated enzyme whereas the kinase domain is the ATP-binding site which catalyses the formation of the phosphorylated intermediate (Serrano 1989). From the results obtained by trypsin treatment and genetic approaches, the plasma membrane H^+-ATPase C-terminal region has a negative regulatory function in plants, acting as an auto-inhibitory domain (Morsomme and Boutry 2000).

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Abbreviations: ATP - adenosine triphosphate; BSA - bovine serum albumin; DTT - dithiothreitol; DR - dune reed; EDTA - ethylenediaminetetraacetic acid; Mes - 2-(*N*-morpholino)-ethanesulfonic acid; PMSF - phenylmethylsulfonyl fluoride; PNPP - *p*-nitrophenyl phosphate; PVP - polyvinylpolypyrrolidone; SR - swamp reed; Tris - *N*-tris(hydroxymethyl)-amino methane.
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[1] Corresponding author; fax: (+86) 10 62590833; e-mail: kunmingchen@163.com

The plant plasma membrane H^+-ATPase is encoded by a multigene family, its gene expression is tissue-specific, and the activity is tightly regulated at transcriptional, translational and enzymatic levels (Sussman 1994). In recent years, important progress has been made concerning the identification and organization of the plasma membrane H^+-ATPase genes, their expression, and also the kinetics and regulation of individual H^+-ATPase isoforms (see review Morsomme and Boutry 2000). Studies performed with both plants and yeasts have shown that the plasma membrane H^+-ATPase is not only regulated by phospholipids, Ca^{2+}, hormones, cGMP, light, and 14-3-3 proteins (Serrano 1989, Briskin 1990, Cooke and Burden 1990, Michelet and Boutry 1995, Suwastika and Gehring 1999, Morsomme and Boutry 2000), but also affected by various environmental factors, such as cold, salt, drought, iron deficiency and γ-radiation (Dong *et al*. 1994, An *et al*. 1999, Rodriguez-Rosales *et al*. 1999, Dell'Orto *et al*. 2000). Given the diversity of biological functions supported by the plasma membrane H^+-ATPases and the multiplicity of factors affecting their activity, the question arises as to whether certain isoforms are specialized for working under specific environmental conditions or in specific cell types, tissues or organs.

Recently, information about the plasma membrane H^+-ATPase in plants subjected to drought stress has also been obtained, although the observations seemed to differ between different studies. Sailerova and Zwiazek (1993) found that osmotic stress inhibited the plasma membrane H^+-ATPase activity from white spruce needles. Qiu (1999a) observed similar results in wheat roots. However, Surowy and Boyer (1991) found that ATPase expression in soybean roots was increased at low water potential. We also observed an increased activity of the plasma membrane H^+-ATPase from wheat leaves under drought stress in the field (Gong *et al*. 2003). However, almost all of these results were obtained under single artificial stress conditions, where the stress imposed to plants was often abrupt and transitory. Conversely, in the natural habitats of plants, adverse environmental factors are hardly present alone, and they are often progressive and continuous. For example, osmotic stress caused by drought in the summer is often accompanied by high-temperature stress (Riccardi *et al*. 1998). It has been found that artificial stresses under laboratory conditions can induce cell damage and many stress-responsive genes do not actually contribute to tolerance because their induction likely reflects stress damage (Bray 1993, Xiong *et al*. 1999, Shinozaki and Yamaguchi-Shinozali 2000). Therefore, studying the expression properties of the plasma membrane H^+-ATPase from naturally occurring ecotypes of plants is very important to understand the tolerance mechanisms of the plants to the long-term drought stress.

Reed (*Phragmites communis* Trinius) is a hydrophytic species whose typical habitats are the fresh and brackish water areas of swamps, riversides and lakesides, and has evolved several ecotypes with resistance to drought, salinity and low temperature (Haslam 1970, Matoh 1988, Zheng *et al*. 2000). In addition to swamp reed (SR), there are three other terrestrial reed ecotypes, heavy salt meadow reed (HSMR), light salt meadow reed (LSMR) and dune reed (DR), growing in the desert regions of northwest China. In the course of our long-term studies, these four reed ecotypes have shown some stable variations of morphological, physiological and genetic characteristics in response to drought and salinity (Wang *et al*. 1998, Zheng *et al*. 2000, Cheng *et al*. 2001, Zhu *et al*. 2001, Chen *et al*. 2003). In this work, two ecotypes of the reed, swamp reed (SR) and dune reed (DR), were used to demonstrate the possible changes of the catalytic process of the plant plasma membrane H^+-ATPase when the hydrophytic reed transferred its habitat from water to drought-prone dunes.

Materials and methods

Plant material and sampling site were described previously (see Chen *et al*. 2004). During July 1 to 3 in 2002, the second leaves from the top of the two reed ecotypes were simultaneously collected at midday and frozen in liquid N_2 until the preparation of the plasma membranes was performed.

Leaf plasma membranes were isolated by the discontinuous sucrose density gradient method according to Qiu and Sun (1998) with modifications. Leaves were ground into power with liquid N_2 and homogenized with precooled extracting buffer [25 mM Tris-Mes, pH 7.5, 1 mM phenylmethylsulfonyl fluoride (PMSF), 15 mM β-mercaptoethanol, 1 mM dithiothreitol (DTT), 3 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ATP, 0.25 M sucrose, 0.5 % polyvinylpolypyrrolidone (PVP) and 0.5 % bovine serum albumin (BSA)]. The homogenate filtered through 2-layers of cheese cloth was centrifuged for 15 min at 13 000 *g*. The supernatant was centrifuged again at 80 000 *g* for 30 min to get a microsomal pellet, which was suspended in a buffer containing 2 mM Tris-Mes, pH 7.2, 1 mM DTT, and 0.25 M sucrose. The microsomal membranes were carefully layered onto a previously prepared discontinuous gradient of sucrose solutions which consisted of 34 % (m/m) sucrose layered over 41 % (m/m) sucrose in 2 mM Tris-Mes (pH 7.2). Then they were centrifuged at 100 000 *g* for 2 h. The fraction at the interface of 34 - 41 % sucrose solution was collected, diluted with suspension buffer, and then centrifuged at 80 000 *g* for 30 min, after which the pellet (plasma membranes) was collected and resuspended in 2 mM Tris-Mes buffer, pH 7.2, containing 1 mM DTT and 0.25 M sucrose. The protein content was determined by the method of Bradford (1976) with BSA as standard.

The purity of plasma membrane was estimated according to the method of Widell and Larsson (1990). Specific inhibitors, including vanadate, azide, molybdate and nitrate, were used, indicating that highly purified plasma membranes were obtained as described previously (Chen *et al*. 2004).

The H^+-ATPase hydrolytic activity of plasma membranes was determined by measuring the release of Pi from ATP according to the method of Qiu (1999b) with modification. The reaction medium contained 3 mM $MgSO_4$, 50 mM KCl, 1 mM NaN_3, 50 mM $NaNO_3$, 0.1 mM Na_2MoO_4, 0.02 % Triton X-100, 25 mM Tris-Mes (pH 6.5), and 10 μg plasma membrane protein in a final volume of 0.5 cm^3. The reaction was started by adding ATP-Na_2 into a final concentration of 3 mM, and progressed for 30 min at 30 °C, after which the reaction was stopped by adding 0.1 cm^3 of 18 % TCA. Then 0.1 cm^3 of 0.56 % SDS was added to prevent any precipitation during inorganic phosphate determination.

The activity of PNPP hydrolysis of plasma membranes was assayed in the same way as ATP hydrolysis, except using PNPP instead of ATP-Na_2.

Trypsin treatment of plasma membrane vesicles was performed as described by Qiu and Zhang (2001). The proteolysis medium contained 25 mM Tris-Mes, pH 7.5, 2 mM DTT, 5 mM EDTA, 0.25 M sucrose, and 50 μg of the plasma membrane protein.

Results

Hydrolytic activities and kinetic parameters of ATP and PNPP hydrolysis by the plasma membrane H^+-ATPase: Plasma membrane H^+-ATPase activity was assayed in the membranes isolated from leaves of the two reed ecotypes, SR and DR, respectively, at different ATP concentrations in the range 0.125 - 2.00 mM (Fig. 1*A*). The kinetic parameters of plasma membrane H^+-ATPase from the two reed ecotypes were calculated based directly on the Michaelis-Menten equation. The data show that K_m for ATP hydrolysis of plasma membrane H^+-ATPase was similar in the two reed ecotypes while V_{max} in the DR was almost 10-fold of that in the SR (Table 1).

The PNPP hydrolysis activity of the plasma membrane H^+-ATPase from the two reed ecotypes was also assayed at different PNPP concentrations in the range 1 - 30 mM (Fig. 1*B*). K_m and V_{max} for PNPP hydrolysis of plasma membrane H^+-ATPase show a great differences between the two reed ecotypes (Table 1). In the DR plants the K_m and V_{max} values were 2- and 10-fold, respectively, of these in the SR plants.

Table 1. Kinetic parameters of ATP and PNPP hydrolysis by the plasma membrane H^+-ATPase (K_m and V_{max}) from the two reed ecotypes, SR and DR. The results are means ± SD of three independent experiments with activities assayed in triplicate. Values followed by different letters differ significantly ($P \leq 0.05$).

Substrate	K_m [mM]		V_{max} [μmol(Pi) mg^{-1}(protein) min^{-1}]	
	SR	DR	SR	DR
ATP	0.28 ± 0.05a	0.24 ± 0.03a	0.19 ± 0.03a	1.89 ± 0.26b
PNPP	3.74 ± 0.52b	8.09 ± 0.71c	0.92 ± 0.13c	9.66 ± 0.65d

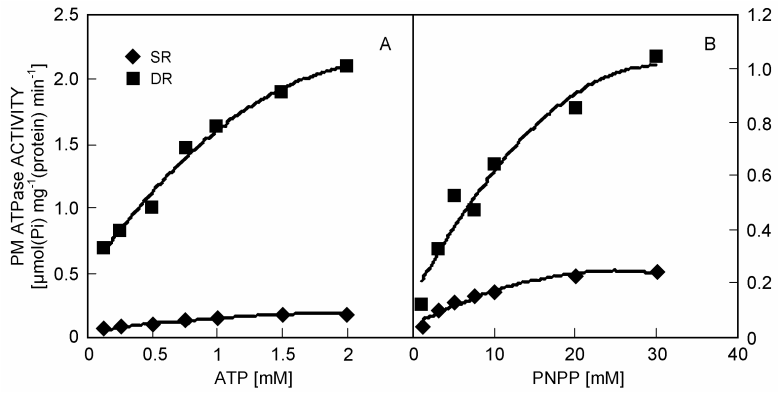

Fig. 1. ATP-dependence of ATP hydrolysis (*A*) and PNPP-dependence of PNPP hydrolysis (*B*) by the plasma membrane (PM) H^+-ATPase from the two reed ecotypes, swamp reed (SR) and dune reed (DR). The data are the means (SD ≤ 10 %) of two independent experiments with each three replicates.

Vanadate is a specific inhibitor of the plasma membrane H^+-ATPase and its action site is at the phosphatase domain of this enzyme (Serrano 1989). In the present work, vanadate obviously inhibited PNPP hydrolysis in both reed ecotypes (Fig. 2*A*), and with increased concentrations of vanadate (0.2 - 2.0 mM), the percentage of inhibition was also progressively increased (Fig. 2*B*). Although the PNPP hydrolysis activity of the plasma membrane H^+-ATPase was markedly different between the two reed ecotypes, the percentage inhibition of PNPP hydrolysis rate was similar in both ecotypes (Fig. 2*B*).

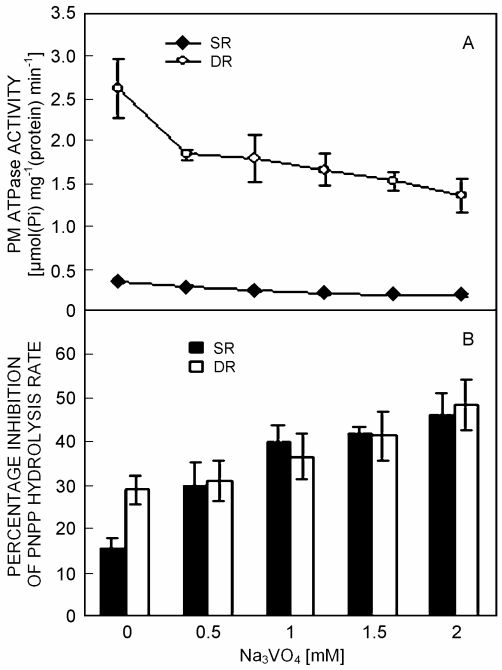

Fig. 2. Inhibitory effect of vanadate on PNPP hydrolysis activity (*A*) and percentage inhibition of PNPP hydrolysis rate (*B*) of the plasma membrane H^+-ATPase from the two reed ecotypes, swamp reed (SR) and dune reed (DR). The means ± SD of three independent experiments each with three replicates.

The pH optimum of plasma membrane H^+-ATPase from different ecotypes of reed: The pH optimum of the plasma membrane H^+-ATPase was 6.5 in both reed ecotypes, SR and DR. However, compared to SR, the activity of the plasma membrane H^+-ATPase from DR was more strongly affected by the reaction medium pH (Fig. 3).

Effect of hydroxylamine on the plasma membrane H^+-ATPase activity: It has been proven that one of the typical properties of the P-type ATPase is the generation of a phosphorylated intermediate by the catalysis of the kinase domain (Vara and Serrano 1983). The phosphorylation site is at the Asp residual of the second motif, forming an acyl-phosphate bond which could be broken by hydroxylamine (Briskin 1990). The ATP hydrolysis activity of the plasma membranes in both reed ecotypes was markedly inhibited by increasing concentrations of hydroxylamine (100 - 500 mM, Fig. 4*A*), and the percentage inhibition of the ATP hydrolysis rate seemed higher in DR than that in SR (Fig. 4*B*).

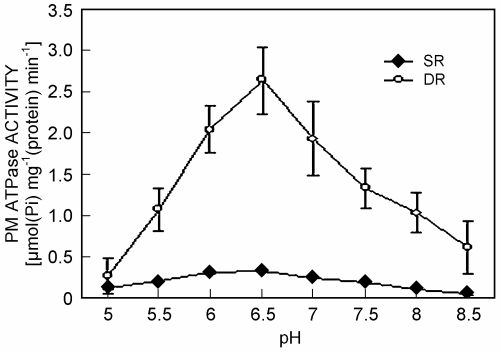

Fig. 3. Effect of the reaction medium pH on the activity of the plasma membrane H^+-ATPase from the two reed ecotypes, swamp reed (SR) and dune reed (DR). The means ± SD of two independent experiments each with three replicates.

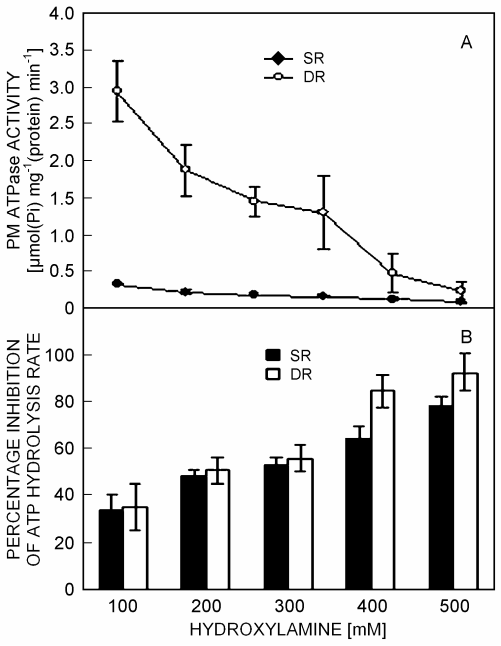

Fig. 4. Inhibitory effect of hydroxylamine on ATP hydrolysis activity (*A*) and percentage inhibition of ATP hydrolysis rate (*B*) of the plasma membrane H^+-ATPase from the two reed ecotypes, swamp reed (SR) and dune reed (DR). The means ± SD of three independent experiments each with three replicates.

Effect of trypsin on ATP hydrolysis of the plasma membrane H^+-ATPase: Trypsin stimulated the ATP hydrolysis activity of the plasma membrane in both reed ecotypes at different trypsin concentrations in the range 0.1 - 5.0 μg cm^{-3}. Compared to SR, the increase of the plasma membrane H^+-ATPase activity with trypsin treat-

ment was higher in DR, and when the value reached the maximum in the two reed ecotypes, respectively, the activity was increased by 7.7 and 15.0 % of the respective controls (no trypsin treatments). Moreover, the trypsin concentration for maximum stimulation was also different, being 3.0 μg cm^{-3} in SR and 5.0 μg cm^{-3} in DR (Fig. 5).

Discussion

The H^+-ATPase, the most abundant protein in the plant plasma membrane, pumps protons from the cytoplasm to the cell exterior using ATP as the energy source, and thus,

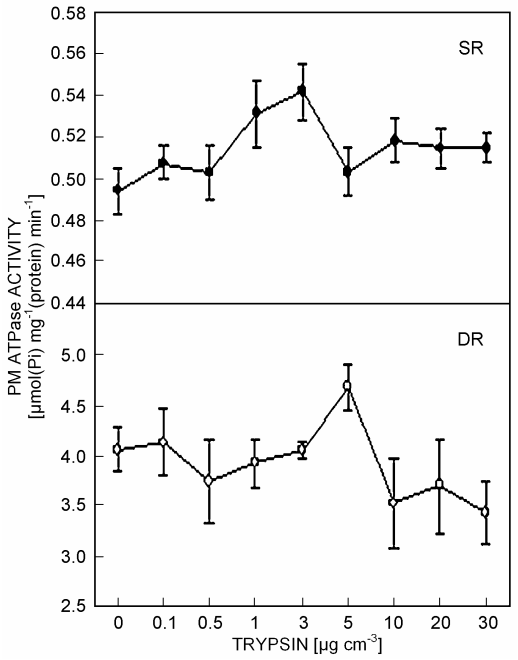

Fig. 5. Effect of trypsin on ATP hydrolysis activity of the plasma membrane H^+-ATPase from the two reed ecotypes, swamp reed (SR) and dune reed (DR). The means ± SD of three independent experiments each with three replicates.

produces an electrochemical gradient across the plasma membrane as the driving force for nutrient uptake (Serrano 1989, Morsomme and Boutry 2000). In recent years, much attention has focused on the identification and organization of the H^+-ATPase genes, their expression, and also the kinetics and regulation of individual H^+-ATPase isoforms, especially under environmental stress conditions such as drought and sanility. Under drought stress conditions, some studies have shown that this enzyme was involved in the response of plants to drought and that some catalytic properties were changed by this environmental factor (Sailerova and Zwiazek 1993, Zhao *et al*. 2000a,b, Gong *et al*. 2003). However, information of the precise mechanism by which the expression and metabolic regulation of the plasma membrane H^+-ATPase is affected by drought stress remained scants, especially for the H^+-ATPase from naturally occurring species.

As described by Morsomme and Boutry (2000), the catalytic cycle proposed for the plasma membrane H^+-ATPase is based mainly on the current models for the mammalian Na^+, K^+-ATPase and Ca^{2+}-ATPases. The general scheme includes two distinct conformational states of the enzyme, termed E1 and E2. When in the E1 state, ATP and proton bind to the enzyme, and then a high energy phosphorylated intermediate is formed and ADP is released. A conformational change then occurs, shifting the enzyme from the E1 to the E2 conformation, followed by the release of the proton to the exterior. Finally, Pi is released and the enzyme returns to E2. The whole catalytic process includes E1 phosphorylation, E1-E2 transition, proton transduction and E2 dephosphorylation. In this catalytic cycle, the processes of ATP-binding and phosphorylation are conducted by the kinase domain, and the dephosphorylation is conducted by the phosphatase domain of the enzyme (Serrano 1989). Therefore, the regulations of the kinase and phosphatase domains are important factors for the enzyme under environmental stresses.

Modifying developmental properties in consequence to environmental changes is the main factor for plant adaptation to special habitats. It has been found that several isoforms of the plasma membrane H^+-ATPase exist in plants, which are differentially expressed and regulated both developmentally and by environmental conditions (Michelet and Boutry 1995, Ballesteros *et al*. 1998). Data from our previous study showed that in droughted wheat plants the pH optimum of the plasma membrane H^+-ATPase was shifted to more alkaline values (from 6.5 to 7.0; Gong *et al*. 2003). In the present work, we found that the pH optimum of the plasma membrane H^+-ATPase in the both reed ecotypes was not changed but the sensitivity of the enzyme to the reaction medium pH seemed to be different (Fig. 3). Compared to SR, the DR exhibited the higher V_{max} value and ATP hydrolysis activity of the plasma membrane H^+-ATPase even though the K_m value for the ATP hydrolysis was almost similar in both reed ecotypes (Fig. 1*A*, Table 1), indicating that a different isoform of the plasma membrane H^+-ATPase may be developed in the DR when reed plants changed their habitats from water to drought-prone dunes. This viewpoint was also supported by the results from the assay of PNPP hydrolysis activity of the plasma membranes from the two reed ecotypes reported here. PNPP is a substrate of phosphatases (Lowry 1957). Qiu (1999b) using soybean hypocotyls demonstrated that the plant plasma membrane H^+-ATPase also had PNPP hydrolysis activity and confirmed that the catalytic process proceeded by the phosphatase domain of the enzyme. In the previous work, we found that drought stress stimulated the PNPP hydrolysis activity of the ATPase and we demonstrated that the change of PNPP

hydrolysis activity by drought was due to the alterations of the catalytic mechanism of the ATPase phosphatase domain (Gong *et al*. 2003). In the present work, the PNPP hydrolysis activity of the plasma membrane H^+-ATPase was greatly higher in DR than that in SR (Fig. 1*B*) and the percentage inhibition of the activity by vanatate, a specific inhibitor of the plasma membrane H^+-ATPase whose action site is at the phosphatase domain of the enzyme, was almost similar (Fig. 2), indicating that the catalytic mechanism of the phosphatase domain of the plasma membrane H^+-ATPase from the different reed ecotypes was different. The results of K_m and V_{max} assays for PNPP hydrolysis in the two reed ecotypes (Table 1) more obviously showed the difference, implying that the regulation of the phosphatase domain of the plasma membrane H^+-ATPase may be involved in adaptation of reed plants to their an extreme habitats such as dunes. Hydroxylamine is an inhibitor of the plasma membrane H^+-ATPase which blocks the formation of the phosphorylated intermediate catalysed by the kinase domain of this enzyme (Vara and Serrano 1983, Briskin 1990). Thus, the difference of sensitivity of the plasma membrane H^+-ATPase to hydroxylamine in the two reed ecotypes (Fig 4) indicated that the regulation of the kinase domain of the enzyme also might be involved in adaptation to the extreme habitat. It has been found that the H^+-ATPase C-terminal region is an auto-inhibitory domain and trypsin treatment can increase the ATP hydrolysis activity of the enzyme (Palmgren *et al*. 1990). The results from trypsin treatment in the two reed ecotypes (Fig. 5) reported here suggested that also the change of the structure or property of the C-terminal end of the plasma membrane H^+-ATPase might be responsible for the adaptation of the plant to the dry habitat. All of these information suggest that, when reed, a hydrophytic plant, transfers its habitat from water to the drought-prone dunes, the structure and catalytic properties of the plasma membrane H^+-ATPase concerning the regulations of the C-terminal region, and the phosphatase and kinase domains were changed. This implies that a different isoform of the plasma membrane H^+-ATPase might be developed in adaptation of the plant to the long-term drought-prone habitat.

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