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The regulation of the plasma membrane redox system and H^+-transport in adaptation of reed ecotypes to their habitats

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Abstract

The redox system and H^+-transport activities in the plasma membranes from two ecotypes of reed (*Phragmites communis* Trin.), named swamp reed (SR) and dune reed (DR) according to their habitats, were investigated. Compared to the SR, the DR possessed the very high rates of NADH oxidation and $Fe(CN)_6^{3-}$ and EDTA-Fe^{3+} reduction when NADH was taken as the electron donor. As NADPH was an electron donor, the rate of NADPH oxidation was also significantly higher in the DR than that in the SR. In addition, the H^+-transport activity in the plasma membranes was also significantly higher in the DR than in the SR.

Additional key words: ATPase activity, dune reed, ferricyanide reduction, NADH oxidation, *Phragmites communis*, swamp reed.

Introduction

It has been documented that the proton electrochemical gradient across the plasma membrane directly or indirectly provides energy for ion and metabolite transport into and out of the plant cell. Besides the plasma membrane H^+-ATPase, generation of this gradient is also dependent on the plasma membrane-associated redox processes (Serrano 1989, Böttger *et al*. 1991, Bérczi and Møller 2000, Morsomme and Boutry 2000). Plasma membrane redox system is an electron transfer chain in the plasma membrane including electron donor oxidases, electron vectors and electron acceptor reductase, *etc*., through which electron is translocated from donor to its acceptor (Böttger *et al*. 1991, Bérczi and Møller 2000). The cytoplasm NADH and NADPH are the natural electron donors, and ferric chelate, ferricyanide, oxygen, and ascorbate free radical have been proposed as the natural electron acceptors (Navas *et al*. 1994, Bérczi and Møller 2000). The plasma membrane redox system is involved in a number of physiological processes such as ion uptake, iron reduction, blue light responses, defence against pathogen attacks, and cell wall synthesis (Møller and Crane 1990, Askwith and Kaplan 1998, Bérczi and Møller 2000). Barr (1991) found that the redox system of plasma membranes from radish was very sensitive to salt stress, which resulted in the inhibition of NADH oxidization, $Fe(CN)_6^{3-}$ reduction and H^+ secretion that eventually affected plant growth. Zhao *et al*. (2000) observed that the rate of NADH oxidation and $Fe(CN)_6^{3-}$ and EDTA-Fe^{3+} reduction were decreased by water stress. Zhao *et al*. (1995) suggested that the plasma membrane redox system might be involved in the regulation of frost hardiness in roots of jack pine seedlings. For our knowledge, the relation of the plasma membrane redox system to the adaptation of plants to their long-term extreme habitats such as drought and salinity has not been explored.

Typical habitats of reed are fresh and brackish water areas of swamps, riversides and lakesides. However, the plant has evolved various ecotypes with adaptation to adverse terrestrial habitats (Haslam 1970, Matoh *et al*.

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Abbreviations: ATP - adenosine triphosphate; BSA - bovine albumin serum; BTP - 1,3-bis[tris-(hydroxymethyl)methylamino] propane; DR - dune reed; EDTA - ethlenediaminetetraacetic acid; Mes - 2-(N-morpholino)ethanesulfonic acid; NADH - reduced nicotinamide adenine dinucleotide; NADPH - reduced nicotinamide adenine dinucleotide phosphate; PMSF - phenylmethylsulfonyl fluoride; PVP - polyvinylpolypyrrolidone; SR - swamp reed; Tris - N-tris(hydroxymethyl)-amino methane.
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1988, Zheng *et al*. 2000). It has been found that there are four reed ecotypes growing in the desert regions of northwest China (Wang *et al*. 1998, Zheng *et al*. 2000, Cheng *et al*. 2001, Zhu *et al*. 2001, Chen *et al*. 2003). In this work, the plasma membrane redox system and H^+-transport activities were investigated in the two ecotypes of reed, common swamp reed (SR) and dune reed (DR), growing in the desert of northwest of China. The variation between the two reed ecotypes that might contribute to their adaptation to the respective habitat was also considered.

Materials and methods

Two reed ecotypes of *Phragmites communis* Trin., referred to as swamp reed (SR) and dune reed (DR) according to the traits of their respective habitats (Wang *et al*. 1998, Zheng *et al*. 2000, Chen *et al*. 2003), grow in Pingchuan Town, Linze County, Gansu Province, China. This region belongs to Linze Research Area, the Cold and Arid Regions Environmental and Engineering Research Institute of Chinese Academy of Sciences (39°4′-24′N, 99°25′-35′E; elevation 1 300 m) and has a typical desert landscape as described previously (Chen *et al*. 2003). The two reed ecotypes were sampled from different habitats at the same region. The SR grows over 2 m deep in pools with 0.35 % salt content in its root zone, whereas the DR grows in sand dunes with 13.7 % water content and only 0.09 % salt content in the root zone. Since all sampling sites are located within a narrow area (about 6.5 km^2), the reed ecotypes under study, although found in varying soil water and salt habitat, share similar meteorological conditions. 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 discontinuous sucrose density 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, 2.5 mM PMSF, 3 mM EDTA, 0.25 M sucrose, and 0.6 % PVP-40), the homogenate filtered through 2-layer 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, 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 % 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 0.25 M sucrose. The protein content was determined by the method of Bradford (1976) with bovine serum albumin (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 was obtained (Table 1).

The oxidation rate of NAD(P)H was assayed by the method of Qiu *et al*. (1994) and Zhao *et al*. (2000). To 1 cm^3 of reaction buffer (10 mM Tris-Mes, pH 8.0, 0.25 M sucrose) was added 0.005 cm^3 of 50 mM NADH or NADPH, 0.01 cm^3 of 100 mM $Fe(CN)_6^{3-}$ and 10 μg plasma membrane protein. Changes of absorbance at 340 nm (*Shimadzu UV-1601* spectrophotometer, Japan) were recorded with the above solution containing no membrane as the blank. Coefficient of absorbance 6.23 mmol cm^{-1} was used to calculate the oxidization rate.

Ferricyanide reduction by plasma membranes was determined with the same reaction solution as that of NADH oxidation. Changes of absorbance at 420 nm were recorded. Coefficient of absorbance 1 mmol cm^{-1} was used for calculating the reduction rate.

The reduction rate of EDTA-Fe^{3+} was assayed with the reaction solution contained 1 cm^3 of 0.25 M sucrose in 10 mM Tris-Mes, pH 8.0, 0.005 cm^3 of 50 mM NADH, 0.01 cm^3 of 1,10-phenanthroline hydrochloride and 0.01 cm^3 of 100 mM EDTA-Fe^{3+}. The reaction was initiated by adding 10 μg plasma membrane protein. Absorbance at 535 nm was recorded with the reaction solution without plasma membrane as the blank. A standard curve prepared using Fe^{2+} was taken to calculate the reduction rate of EDTA-Fe^{3+}.

The H^+-ATPase hydrolytic activity of plasma membranes was determined by measuring the release of Pi from ATP according to the method of Qiu (1999) 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 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 H^+-transport activity by plasma membrane was

assayed according to the method of Klobus and Buczek (1995) with modifications. The reaction medium contained 25 mM BTP-Mes, pH 8.0, 0.33 M sorbitol, 3 mM ATP-Na_2, 3 mM $MgSO_4$, 50 mM KCl, 0.1 % BSA and 10 μM acridine orange. The reaction was started by the addition of ATP. Changes in absorbance of acridine orange at 495 nm were recorded.

One-way analysis of variance was used for comparisons between the means by Fisher's PLSD test with the *DSTP*-statistical package (Feng and Tang 1997).

Results

Highly pure plasma membranes from the two reed ecotype leaves were obtained (Table 1). By using diagnostic inhibitors for different phosphohydrolase activities, the degree of contamination by membranes of other organ was characterized. Total ATPase activity was mostly inhibited by vanadate (inhibitor of P-type H^+-ATPases) in both reed ecotypes, SR and DR, but by azide (inhibitor of F-type H^+-ATPases), nitrate (inhibitor of V-type H^+-ATPases), or molybdate (inhibitor of acid phosphatases), no obvious inhibition of the total ATPase activity was observed, indicating minor contaminations of the plasma membrane preparations by other membrane fractions. The vanadate-sensitive ATPase activity was almost similar whether in absence or presence of 0.05 % Triton X-100 in both reed ecotypes, respectively (Table 1). The small latency of vanadate-sensitive ATPase in both reed ecotypes showed that the sidedness of the plasma membrane vesicles was similar in the two reed ecotypes.

Table 1. Assays of purity and latency of the plasma membranes from the two reed ecotypes, swamp reed (SR) and dune reed (DR). The activity of the vanadate-sensitive ATPase [mmol(Pi) g^{-1}(protein) min^{-1}] was assayed in 25 mM Tris-Mes, pH 6.5, 3 mM $MgCl_2$, 50 mM KCl and 3 mM ATP. Means ± SD of two independent experiments with activities assayed in triplicates. Values followed by different letters differ significantly ($P \leq 0.05$) according to Fisher's PLSD test.

Incubation medium		ATPase activity	
Inhibitors [mM]	Triton X-100	SR	DR
None	None	0.274 ± 0.034ab	3.23 ± 0.20d
None	0.05 %	0.257 ± 0.023ab	3.54 ± 0.43d
$NaNO_3$ (50)	0.05 %	0.292 ± 0.023a	3.85 ± 0.34d
NaN_3 (1.0)	0.05 %	0.231 ± 0.014b	3.55 ± 0.36d
Na_2MO_4 (0.1)	0.05 %	0.262 ± 0.031ab	3.54 ± 0.46d
Na_3VO_4 (1.0)	0.05 %	0.165 ± 0.028c	1.67 ± 0.39e

When ferricyanide or NADH was present separately in the reaction medium, very low, if not any redox activities were observed in both reed plasma membranes. However, when ferricyanide and NADH were both present in the reaction medium, NADH was rapidly oxidized and ferricyanide was quickly reduced (Table 2). These results suggest that a redox system may exist in the plasma membranes of both reed ecotypes.

The effects of two inhibitors of respiratory chain, *i.e.* KCN and NaN_3 (Szabó-Nagy and Erdei 1993) on the NADH oxidation and ferricyanide reduction were also investigated. No significant inhibition was found in both

Table 2. Effect of different mediums on the ferricyanide reduction [nmol mg^{-1}(protein) min^{-1}] and NADH oxidation rate [nmol mg^{-1}(protein) min^{-1}] in the two reed ecotypes, SR and DR. Means ± SD were calculated from 4 - 5 replicates from two independent experiments. Values followed by different letters in each column differ significantly ($P \leq 0.001$).

Ecotype	$Fe(CN)_6^{3-}$	NADH	NADH + $Fe(CN)_6^{3-}$ Ferricyanide reduction rate	NADH oxidation rate
SR	0a	36.2 ± 4.2a	1014.3 ± 75.8a	2015.4 ± 86.3a
DR	0a	58.6 ± 10.1b	9398.1 ± 2510.4b	17745.5 ± 1332.3b

Table 3. Effect of inhibitors on the plasma membrane redox activity of the two reed ecotypes, SR and DR. Means ± SD of 5 - 6 replicates from two independent experiments. Values followed by different letters differ significantly ($P \leq 0.05$).

Inhibitor	NADH oxidation rate [mmol g^{-1}(protein) min^{-1}]		$Fe(CN)_6^{3-}$ reduction rate [mmol g^{-1}(protein) min^{-1}]	
	SR	DR	SR	DR
None	0.99 ± 0.08a	9.11 ± 0.30b	1.92 ± 0.17a	17.59 ± 1.49b
KCN (0.1 mM)	0.96 ± 0.09a	9.04 ± 0.93b	2.00 ± 0.05.4a	17.59 ± 1.35b
NaN_3 (1.0 mM)	0.97 ± 0.06a	10.31 ± 0.82b	1.91 ± 0.16a	18.21 ± 2.76b

Table 4. Redox activities [mmol g^{-1}(protein) min^{-1}] in the plasma membranes of the two reed ecotypes, SR and DR. Means ± SD of 6 - 8 replicates from two independent experiments. Values followed by different letters in each row differ significantly ($P \leq 0.001$).

	SR	DR
NADH oxidation	1.08 ± 0.07a	9.13 ± 2.41b
NADPH oxidation	0.26 ± 0.03a	10.70 ± 2.21b
$Fe(CN)_6^{3-}$ reduction	2.23 ± 0.16a	17.59 ± 0.90b
EDTA-Fe^{3+} reduction	0.044 ± 0.004a	0.144 ± 0.024b

Table 5. The H^+-transport activity [ΔA_{495} mg^{-1}(protein) min^{-1}] of the plasma membranes from the two reed ecotypes, SR and DR, and its inhibition by vanadate. Results are given as the absorbance changes during the first minute of reaction. Means ± SD of 6 - 8 replicates from two independent experiments. Values followed by different letters differ significantly ($P \leq 0.001$).

Incubation medium	SR	DR
Control	0.80 ± 0.13a	10.44 ± 2.18c
Na_3VO_4 (250 μM)	0.30 ± 0.10b	3.23 ± 0.76d

reed ecotypes, SR and DR, indicating that the characteristics of the plasma membrane redox system were different from the respiratory chain of mitochondria and the plasma membranes obtained here were highly purified (Table 3).

The redox activities of the plasma membranes from the two reed ecotypes were significantly different (Table 4). The DR exhibited a higher redox activity of the plasma membranes as compared to the SR. Taking ferricyanide as an electron receptor, the oxidation rate of NADH and NADPH in the DR was 8.49- and 40.47-fold of those in SR, respectively. When NADH was used as an electron donor, the reduction rates of ferricyanide and EDTA-Fe^{3+} were also higher in the DR than the SR, those in the former being 7.88- and 3.25-fold of those in the SR, respectively. One should pay more attention to the fact that the rate of NADH oxidation was significantly higher than that of NADPH oxidation in the SR while such difference was not obviously observed in the DR.

The H^+-transport activity of the plasma membranes in the DR was significantly higher being 13.0-fold of that in the SR. The plasma membrane H^+-transport activity was obviously inhibited by vanadate in both reed ecotypes, indicating that the activity was dependent on the plasma membrane ATPase (Table 5).

Discussion

Plants possess the ability to acclimate to a variety of environmental stresses. As described previously, although the reed ecotypes shared similar meteorological conditions, they differed markedly in growth and development (Wang *et al*. 1998, Zheng *et al*. 2000, Chen *et al*. 2003).

Plasma membrane redox system is another energizing system and might be involved in establishment of proton gradient across the plasma membrane together with the plasma membrane H^+-ATPase (Böttger *et al*. 1991, Szabó-Nagy and Erdei 1993). Plants, in general, have a plasma membrane bound standard redox system which can reduce extracellular electron acceptors (Barr *et al*. 1985, Crane *et al*. 1985). The standard system is constitutive and ubiquitous in the plasma membrane of all plants and can reduce most ferricyanide but not other ferric chelates (Szabó-Nagy and Erdei 1993). In addition, another redox system, named "turbo system", was also found in some plants, and this system uses ferric chelates as substrate (Grabov *et al.* 1993; Rubinstein and Luster 1993). In the present work, when NADH was taken as the electron donor, the ferricyanide reduction activity in both reed ecotypes was very high and the DR possessed significantly higher redox activities than the SR; in addition, EDTA-Fe^{3+} could also serve as an electron acceptor in the reed ecotypes although the reduction rate was relatively slow (Table 4). That is to say, besides the "standard system", the "turbo system" also exists in the graminaceous plant.

The structure, components and function of the plasma membrane are greatly affected by environmental stresses. Using plasma membranes purified from water-stressed wheat roots, Qiu *et al*. (1994) found that the rates of NAD(P)H oxidation and $Fe(CN)_6^{3-}$ and EDTA-Fe^{3+} reduction were decreased. Gong *et al*. (1999) also observed that the ability of plasma membranes to reduce $K_3Fe(CN)_6$ was decreased both *in vivo* and *in vitro* under water stress. Zhao *et al*. (1995) found that cold acclimation increased the plasma membrane redox activities of roots of jack pine seedlings and suggested that the increase might be related to the increased frost hardiness. As to salt stress, Barr (1991) found that the plasma membrane redox system was very sensitive to artificial salt stress, which resulted in the inhibition of NADH oxidization, $Fe(CN)_6^{3-}$ reduction and H^+ secretion. However, in the present work, the very high activity of plasma membrane redox system was found in the DR which grows in the long-term drought-prone habitat located in the desert region of northwest of China (Table 4). For natural occurring varieties of plants, the major factor for adaptation to special habitat is modifying the developmental properties in consequence to environmental transition. Reed, a hydrophytic species, can adapt to adverse terrestrial habitats such as drought and salinity.

When translocated from water to terrestrial habitats, some metabolic changes occur to couple the changed environment. Results reported here showed that, using NADH as an electron donor, the rate of NADH oxidation, $Fe(CN)_6^{3-}$ reduction were significantly higher in the DR as compared to those in the SR (Table 4), indicating that an up-regulation of the plasma membrane redox system activity was involved in adaptation of the reed ecotype to its long-term drought-prone habitat. In addition, the markedly higher rate of EDTA-Fe^{3+} reduction in the DR than in the SR suggests that the activated "turbo system" was also responsible for drought resistance. Generally, with ferricyanide, NADH as an electron donor is more efficient than NADPH as electron donor in the plasma membranes *in vitro* (Bérczi and Møller 2000). Interesting enough, in our results this was only viewed in the SR but not in the DR (Table 4). In the DR NADH and NADPH seemed sharing similar efficiency as electron donor. The very high rate of NADPH oxidation in the plasma membranes from the DR might be related to plant resistance to drought stress.

Previous studies have suggested that the redox system of the plasma membrane might be involved in the formation or regulation of the proton transfer (Klobus and Buczek 1995). In the present work, similar to activity of the plasma membrane redox system, the significantly high activity of the plasma membrane H^+-transport in the DR (Table 5), also suggests an up-regulation of the H^+-transport activity might be also due to the high redox activity in the reed ecotype. Klobus and Buczek (1995) claimed that NADH-ferricyanide oxidoreductase participates in the proton transport rather indirectly, by regulation of H^+-ATPase activity. Zhao *et al*. (1995) also suggested that the maintenance of redox activity would not only facilitate ion uptake but also play an important role in preventing the inactivation of many transport proteins. It has been inferred that one of the functions of the plasma membrane redox system might be keeping the SH-groups of membrane transport proteins in a reduced state, thus preventing their inactivation by oxidation (Kochian and Lucas 1991). Therefore, since the activity of the plasma membrane H^+-ATPase was also comparatively much higher in the DR than in the SR (Table 1), results reported here indicated that the high H^+-transport activity of the plasma membrane in the DR might be resulted from the up-regulation of the H^+-ATPase activity of the plasma membrane by the high redox activity in the reed ecotype plants.

In summary, when reed, a hydrophytic species, changed its habitat from water to drought-prone dune, the plasma membrane redox system activity became up-regulated. This up-regulation might contribute to the high resistance or tolerance of the plant to its long-term drought-prone habitat. The redox system including the "standard system" and the "turbo system" functions in the plant drought resistance might be due to the up-regulation of the plasma membrane H^+-transport activity mainly caused by the activation of plasma membrane H^+-ATPase in the reed plants.

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