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Increased Polyamines Conjugated to Tonoplast Vesicles Correlate with Maintenance of the H^+-ATPase and H^+-PPase Activities and Enhanced Osmotic Stress Tolerance in Wheat

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Abstract

The effects of osmotic stress on H^+-ATPase and H^+-PPase activities and the levels of covalently conjugated polyamines (CC-PAs) and noncovalently conjugated polyamines (NCC-PAs) were investigated using tonoplast vesicles isolated from the roots of wheat (*Triticum aestivum* L.) seedlings differing in drought-tolerance. The results showed that after polyethylene glycol (PEG) 6,000 (−0.55MPa) treatment for 7 days, seedling leaf relative water content (LRWC), relative dry weight increase rate (RDWIR) and root H^+-ATPase and H^+-PPase activities from the drought-sensitive cultivar Yangmai No. 9 decreased more markedly than those from the drought-tolerant cultivar Yumai No. 18. At the same time, the increase of the NCC-spermidine (NCC-Spd) and CC-putrescine (CC-Put) levels in root tonoplast vesicles from Yumai No. 18 was more obvious than that from Yangmai No. 9. Exogenous Spd treatment alleviated osmotic stress injury to Yangmai No. 9 seedlings, coupled with marked increases of tonoplast NCC-Spd levels and H^+-ATPase and H^+-PPase activities. Treatments with methylglyoxyl bis (guanyl hydrazone) (MGBG), an inhibitor of S-adenosylmethionine decarboxylase (SAMDC), and phenanthrolin, an inhibitor of transglutaminase (TGase), significantly inhibited the osmotically induced increases of NCC-Spd and CC-Put levels, respectively, in root tonoplast vesicles from Yumai No. 18 seedlings. Both MGBG and phenanthrolin treatments markedly promoted osmotically induced decreases of tonoplast H^+-ATPase and H^+-PPase activities and osmotic stress tolerance of seedlings of this cultivar. These results suggest that the NCC-Spd and CC-Put present in tonoplast vesicles isolated from wheat seedling roots might enhance the adaptation of seedlings to osmotic stress via maintenance of tonoplast H^+-ATPase and H^+-PPase activities.

Key words: Osmotic stress; Polyamines; Tonoplast H^+-ATPase; Tonoplast H^+-PPase; wheat (*Triticum aestivum* L.)

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Introduction

Vacuoles in plant cells play a crucial role in plant responses to various stresses. Vacuoles also serve as sensor sites to determine stress levels and trigger downstream events that allow for the physiological adaptation to the stress (Dietz and others 2001). Two major tonoplast proteins, the H^+-ATPase and H^+-PPase, have been extensively studied and information about their molecular properties has accumulated over the past decade.

Drought is a worldwide problem that threatens food supplies, and the response of plants to drought stress has been extensively investigated (Zhang and Outlaw 2001; Liu and others 2003). However, research into the relationship between water stress and the activities of H^+-ATPase and H^+-PPase in the tonoplast has revealed variable effects (Sanchez-Nieto and others 1998; Qiu 1999; Qiu and Zhang 2000). Shantha and others 2001 found that osmotic shock produced a large vacuolar alkalinization and a decreased pH gradient across the tonoplast in the roots of a relatively osmotically sensitive cultivar of maize, whereas the roots of a relatively osmotically insensitive cultivar of pear millet were able to maintain the pH gradient across the tonoplast with marginal vacuolar alkalinization. This resistance to osmotic stress might be attributed to the sustained activity of tonoplast H^+-pumps. However, Wang and others (2001) showed that H^+-ATPase activity in tonoplast vesicle fractions from polyethylene glycol (PEG)-treated *Suaeda salsa* plants was not significantly different from values obtained from controls. Vera-Estrella and others, (1999) also suggested that osmotic stress produced no changes in H^+-ATPase activity in a salt-tolerant cell line prepared from *Mesembryanthemum crystallinum* plants. The activity of H^+-PPase in a cell line isolated from the non-halophyte, *Dacus carota*, was markedly stimulated by salt treatment, but was unaffected by sorbitol-induced osmotic stress (Colombo and Cerana 1993). However, the data obtained from membrane vesicles of PEG-treated *Suaeda salsa* plants indicated that osmotic stress markedly decreased H^+-PPase activity 3-fold after 8 d of treatment (Wang and others 2001). Such contradictory effects of osmotic stress on H^+-pumping activities might be attributed to different physiological functions of these enzymes in different tissues at different developmental stages. So, the response of both H^+-ATPase and H^+-PPase activities to osmotic stress needs to be further investigated to arrive at a clear conclusion of the relationship between these two enzyme activities and osmotic stress.

Polyamines (PAs) are biologically ubiquitous aliphatic amines that are implicated in many aspects of growth and development in a wide range of organisms (microorganisms, animals, plants, and so on). The common PAs include putrescine (Put), spermidine (Spd), spermine (Spm) and so on S-adenosylmethionine decarboxylase (SAMDC) catalyzes the conversion of S-adenosylmethionine to decarboxylated S-adenoylmethionine, a donor of an aminopropyl moiety to Put and Spd. The synthesis of Spd and Spm is mainly regulated at the level of SAMDC. Methylglyoxyl bis (guanyl hydrazone) (MGBG) is a potent inhibitor of SAMDC (Slocum 1991). Due to their cationic nature at physiological pH (Kumer and others 1997), PAs can interact with macromolecules by hydrogen bonding, ionic bonding, electrostatic and hydrophobic interactions and form noncovalently conjugated PAs (NCC-PAs) in the membrane (Feuerstein and Martin 1989). These interactions allow PAs to play an important role in structure, replication and transcription of DNA, and in stabilizing the function of the bio-membrane (Galston and Kaur-Sawhney 1995). The interaction of PAs with ion channels has been reviewed by Williams (1997). Dobrovinskaya and others (1999) showed that vacuolar ion channels were inhibited by PAs. Liu and others (2000) suggested that polyamines target KAT_1-like channels in guard cells and modulate stomatal movements, providing a link among stress conditions, polyamine levels, and stomatal regulation. Transglutaminase (TGase, EC 2.3.2.13) is a key enzyme that links PAs to endo-glutamines of specific proteins, forming protein-Glu-PA that plays an important role in the posttranslational modifications of proteins (Serafini-Fracassini 1995). The results of Del Duca and others (1995) suggested that polyamines might have an important function in chloroplasts both in their free form and by covalently linking to proteins. However, to our knowledge, the correlation among the H^+-ATPase, H^+-PPase activities and the levels of NCC-PAs and CC-PAs in the tonoplast under osmotic stress remains to be demonstrated.

In the experiments presented here, we detected H^+-ATPase and H^+-PPase activities and the two conjugated PAs (NCC-PAs, CC-PAs) in tonoplast vesicles purified from roots of both a drought-tolerant cv. and a drought-sensitive cv. of wheat under PEG osmotic stress. A possible relationship between these two enzymes and the function of the two conjugated PAs is discussed.

Materials and Methods

Plant Material and Treatments

Two wheat (*Triticum aestivum* L.) cultivars (Yumai No. 18 and Yangmai No. 9) were used as materials. Yumai No. 18 (F_1 generation of Zhengzhou No. 764 and Yanshi No. 4) grows well in drought in the North China ecotope, whereas Yangmai No. 9 (F_1 generation of Yangjian No. 3 and Yangmai No. 5) grows well in the rainy Southeast China ecotope (Zhuang 2003). Yumai No. 18 cv. is drought-tolerant, whereas Yangmai No. 9 cv. is drought-sensitive (Liu and others 2004). The seeds of the two cultivars were surface-sterilized in 0.1% $HgCl_2$ (w/v) for 5 min, rinsed with tap water, and germinated in plastic pots containing sand. The pots were put into half-strength Hoagland solution, which was renewed every 2 d. They were placed in a controlled environment chamber (Model 1915-2, SHELLAB USA) with a temperature of 20°C/10°C (day/night) and 14 h photoperiod at a quantum flux density of 200 μm mol m^2s^{-1} from cool-white fluorescent lamps.

When the extension of the second leaf was completed, the seedlings were treated with Hoagland's solution containing PEG (−0.55 MPa), PEG (−0.55 MPa) + Spd (1 mM), PEG (−0.55 MPa) + MGBG (1 mM) or PEG (−0.55 MPa) + o-Phen (1 mM). Control seedlings were kept in Hoagland's solution without PEG, Spd, MGBG or o-Phen. All the nutrient solutions mentioned above were replaced with fresh solution every 2 d. After treatment for 7 d, the wheat seedlings were sampled.

Reagent grade Put, Spd, Spm, MGBG and o-Phen were obtained from the Sigma Chemical Co. (P.O. Box 14508, St. Louis, MO 63178 USA).

Determination of Leaf Relative Water Content

Leaf relative water content (LRWC) was calculated from the following formula: $LRWC = (W_f - W_d)/(W_t - W_d) \times 100\%$, where W_f, W_d, and W_t represents the fresh weight, dry weight and saturation weight, respectively, using the second fully expanded leaf of each seedling as sample.

Determination of Relative Dry Weight Increase Rate of Wheat Seedlings

Growth rate (GR), on the basis of dry weight, was calculated from the following formula: $GR = (W_a - W_b)/W_b$, where W_a and W_b represents the dry weight of seedling after 7 d and 0 d treatment, respectively. Relative dry weight increase (RDWIR) under each different treatment was calculated from the following formula: RDWIR = (GR of treatment/GR of control) × 100%.

Isolation of Tonoplast Vesicles and Assays of H^+-ATPase and H^+-PPase Activities

Tonoplast vesicles were isolated according to the methods of Ballesteros and others (1996), Suzuki and Kanayama (1999), and Chen and others (1999) with minor modifications. Fifteen g fresh weight of roots were homogenized in 30 ml ice-cold medium containing 250 mM sorbitol, 125 mM KCl, 5 mM EGTA, 2.5 mM $K_2S_2O_5$, 2 mM PMSF, 1.5% (w/v) PVP, 0.1% (w/v) BSA, 1 mM DTT, and 50 mM Hepes-Tris (pH 7.6). The homogenate was filtered through 4 layers of cheesecloth and then differentially centrifuged in steps at 4°C: 800 × *g* for 10 min (pellet discarded), 1,000 × *g* for 15 min (pellet discarded), and 50,000 × *g* for 30 min (pellet recovered). The pellet was gently resuspended in 3 ml medium containing 0.3 M sucrose, 10 mM KCl, 1 mM EGTA, 2 mM DTT and 50 mM Hepes-Tris (pH 7.8), designated as SUC. Then, 2 ml medium containing 0.25 M sorbitol, 1 mM EGTA and 5 mM Hepes-Tris (pH 7.3), designated as SOR, were layered onto 3 ml of SUC and centrifuged at 100,000 × *g* for 2 h. Vesicle bands at the SOR/SUC interface were collected and stored at −80°C until use.

The purity of the vesicles was estimated according to the method of Widell and Larsson (1990), with vanadate, nitrate and azide as inhibitors. The tonoplast H^+-ATPase belongs to the V-type ATPase family which is characterized by inhibition by nitrate, the H^+-ATPase in plasma membrane and mitochondria belong to P-type and F-type ATPases, and these show inhibition by vanadate and azide, respectively. In the present study, different H^+-ATPase activities in the isolated vesicles were assayed in the presence or absence of these inhibitors. Nitrate inhibited the enzyme activity by more than 75%, but vanadate and azide reduced the enzyme activity by less than 2%, demonstrating that the vesicle band at the SOR/SUC interface was enriched in tonoplasts. Determination of H^+-ATPase and H^+-PPase activities was conducted according to the procedure described by Zhang and Liu (2002) with minor modifications. The mixture for the assay of tonoplast H^+-ATPase activity contained 30 mM Hepes-Tris (pH 7.5), 50 mM KCl, 3 mM $MgSO_4$, 0.1 mM ammonium molybdate, 0.25 mM Na_3VO_4, 2 mM ATP-Na_2, and 100 μl of tonoplast vesicles (containing 40–63 μg protein from variously treated

roots). The mixture for the assay of tonoplast H^+-PPase activity contained 30 mM Hepes-Tris (pH 8.0), 50 mM KCl, 2 mM $MgSO_4$, 50 mM KNO_3, 0.2 mM ammonium molybdate, 0.30 mM Na_3VO_4, 2 mM Na_4PPi, and 100 µl of tonoplast vesicles. ATP-Na_2 (or Na_4PPi) was used to start the reaction. After 20 min of reaction at 37°C, 50 µl of TCA was added to stop the reaction. The amount of Pi released from ATP (or PPi) hydrolysis was determined by the method of Ohnishi and others (1975).

Isolation and Determination of Membrane Protein

Triton X-100 (10% (v/v) stock solution) was added to the partialy purified tonoplast vesicle fraction until the terminal concentration amounted to 1% (v/v) at R.T. The solution was sonicated twice for 30 s by means of an ultrasonic disintegrator (BRANSON USA, Model S-150D 100-w), kept on an ice bath for 30 min, and then centrifugated at 100,000 × *g* for 30 min at 4°C (Zhao and others 2000). The supernatant was taken as soluble membrane protein sample. Protein content was determined by the Bradford (1976) method, with BSA as standard. In the present study, the protein concentration was at 6 mg/ml.

PA Analysis

Two conjugated PAs were analyzed according to the method of Sharma and Rajam (1995) with minor modifications. Perchloric acid (PCA) [10% (v/v) stock solution] was added to the soluble membrane protein sample prepared above until the terminal concentration amounted to 5% (v/v), and the sample was centrifugated at 27,000 × *g* for 40 min. The pellet was resuspended in 5% (v/v) PCA, then mixed with 12 N HCl in an equal volume, hydrolyzed at 110°C for 24 h, and desiccated at 70°C after being filtered. Finally, the pellet was dissolved in an equal volume of 5% (v/v) PCA to obtain the solution containing the CC-PAs. By adding PCA [10% (v/v) stock solution] directly to the prepared membrane vesicles until the terminal concentration amounted to 5% (v/v) and centrifuging at 27,000 × *g* for 40 min, the NCC-PAs in membrane vesicles were dissolved in the supernatant. The PAs in the two solutions mentioned above were derivatized with benzyol chloride by the method of DiTomaso and others (1989) and measured by HPLC. Ten µl of methanol (Merck KGaA, Germany)-redissolved samples were injected into a fixed 20 µl loop, loaded onto 3.9 mm by 150 mm, 4 µm particle size C_{18} reverse-phase column (Waters, USA). Samples were eluted from the column by a Perkin-Elmer Series 410 pump at room temperature with a flow rate of 0.5 ml min^{-1}. Polyamine peaks were detected by a Perkin-Elmer LC-95 absorbance detector at 254 nm. The internal standard was 1,6-hexanediamine.

In vitro, the Direct Effect of Spermidine on H^+-ATPase and H^+-PPase Activities of Isolated Tonoplast Vesicles

Root tonoplast vesicles isolated from control wheat seedlings were preincubated *in vitro* in the reaction mediums for H^+-ATPase and H^+-PPase activity assays described above into which Spd (10 mM stock solution) was added until the terminal concentration amounted to 1 mM, at 37°C for 10 min. Then ATP (or PPi) was added into the mediums to start the reaction.

Statistical Analysis

Data were analyzed using SPSS10.0 software (SPSS Inc. Headquarters, 2335 Wacker Drive, Chicago, IL 60606, USA). All the values reported in this paper are the means ± SE of 3 independent experiments. Significant difference among means was determined by Duncan's multiple range test at $p < 0.05$.

Results

Effects of PEG, Spd, MGBG, and o-Phen on Leaf Relative Water Content and Relative Dry Weight Increase Rate of Seedlings

Osmotic stress induced decreases of RDWIR and LRWC in seedlings of the two wheat cultivars tested, Yangmai No. 9 (drought-sensitive) and Yumai No. 18 (drought-tolerant), but these changes in the former were more apparent than those in the latter (Figure 1). These results showed that Yumai No. 18 is more tolerant to PEG-induced osmotic stress than Yangmai No. 9. This PEG-related osmotic injury to Yangmai No. 9 cv. was alleviated with exogenous Spd treatment, as judged by increases of LRWC and RDWIR (Figure 1), whereas exogenous Spd only slightly affected the response of Yumai No. 18 cv. to osmotic stress (Figure 1). Osmotic stress injury to Yumai No. 18 cv. was aggravated more significantly with MGBG or o-Phen treatment than that to Yangmai No. 9 cv. (Fig. 1), as judged by larger decreased ranges of LRWC and RDWIR.

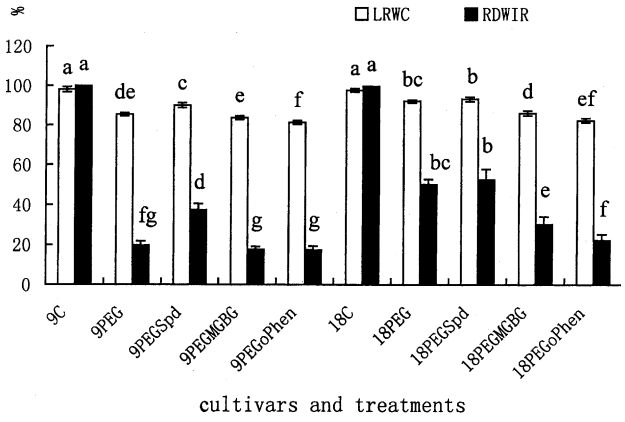

Figure 1. Effects of PEG, PEG+Spd, PEG+MGBG and PEG+o-Phen on LRWC and RDWIR of wheat seedlings. Wheat seedlings were treated with PEG (−0.55 MPa), PEG (−0.55 MPa)+Spd (1 mM), PEG (−0.55 MPa)+MGBG (1 mM) or PEG (−0.55 MPa)+o-Phen (1 mM) for 7 d. Control seedling roots were kept in Hoagland's solution without PEG, Spd, MGBG or o-Phen. The second fully expanded leaves were sampled for LRWC and whole seedlings were sampled for RDWIR. Error bars indicate SE (n = 9). Means with different letters (a ∼ g) are significantly different at $p < 0.05$ based on Duncan's multiple range test. 9C: Yangmai No. 9 Control; 9PEG: Yangmai No. 9 PEG; 9PEGSpd: Yangmai No. 9 PEG+Spd; 9PEGMGBG: Yangmai No. 9 PEG+MGBG; 9PEGoPhen: Yangmai No. 9 PEG+o-Phen; 18C: Yumai No. 18 Control; 18PEG: Yumai No. 18 PEG; 18PEGSpd: Yumai No. 18 PEG+Spd; 18PEGMGBG: Yumai No. 18 PEG+MGBG; 18PEGoPhen: Yumai No. 18 PEG+o-Phen.

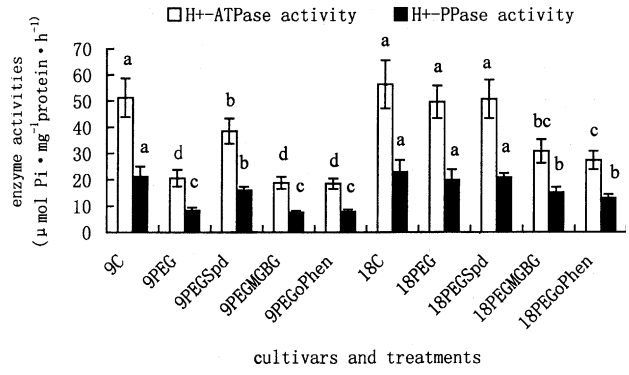

Figure 2. Effects of PEG, PEG+Spd, PEG+MGBG and PEG+o-Phen on the activities of H^+-ATPase and H^+-PPase in tonoplast vesicles isolated from wheat seedling roots. Wheat seedlings were treated with PEG (−0.55 MPa), PEG (−0.55 MPa)+Spd (1 mM), PEG (−0.55 MPa)+MGBG (1 mM) or PEG (−0.55 MPa)+o-Phen (1 mM) for 7 d. Control seedling roots were kept in Hoagland's solution without PEG, Spd, MGBG or o-Phen. The roots were sampled for isolation of tonoplast vesicles. H^+-ATPase and H^+-PPase activities of tonoplast vesicles were determined. Error bars indicate SE (n = 9). Means with different letters (a ∼ d) are significantly different at $p < 0.05$ based on Duncan's multiple range test. 9C: Yangmai No. 9 Control; 9PEG: Yangmai No. 9 PEG; 9PEGSpd: Yangmai No. 9 PEG+Spd; 9PEGMGBG: Yangmai No. 9 PEG+MGBG; 9PEGoPhen: Yangmai No. 9 PEG+o-Phen; 18C: Yumai No. 18 Control; 18PEG: Yumai No. 18 PEG; 18PEGSpd: Yumai No. 18 PEG+Spd; 18PEGMGBG: Yumai No. 18 PEG+MGBG; 18PEGoPhen: Yumai No. 18 PEG+o-Phen.

Effects of PEG, Spd, MGBG, and o-Phen on H^+-ATPase and H^+-PPase Activities in Root Tonoplast Vesicles of Wheat Seedlings

Under PEG-induced osmotic stress, H^+-ATPase and H^+-PPase activities in tonoplast vesicles from wheat seedling roots of cv. Yangmai No. 9 decreased much more markedly than those of Yumai No. 18 (Figure 2). In the seedlings of Yangmai No. 9, exogenous Spd inhibited the PEG-induced decrease of H^+-ATPase and H^+-PPase activities (Figure 2). In contrast, exogenous MGBG or o-Phen treatment promoted the PEG-induced decreases of H^+-ATPase and H^+-PPase activities in root tonoplasts of Yumai No. 18 seedlings (Figure 2).

Effects of PEG, Spd and MGBG on the Levels of Noncovalently Conjugated Polyamines of Root Tonoplast Vesicles from Wheat Seedlings

Under PEG-induced osmotic stress, the levels of NCC-Put and NCC-Spd increased in tonoplast vesicles isolated from the roots of both wheat cultivars. However, NCC-Spd levels in PEG-treated Yumai No. 18 increased much more markedly than those in PEG-treated Yangmai No. 9. In contrast, NCC-Put level in PEG-treated Yumai No. 18 did not increase as much as that in PEG-treated Yangmai No. 9 (Table 1).

With exogenous Spd treatment, the level of tonoplast NCC-Spd increased substantially in PEG-treated Yangmai No. 9 cv. seedling roots but the increase was slight in PEG-treated Yumai No. 18 cv. MGBG treatment led to a significant reduction in the level of NCC-Spd in PEG-treated Yumai No. 18 cv. At the same time, exogenous Spd or MGBG affected the NCC-Put level slightly in both PEG-treated wheat cv. seedlings. With regards to the ratio of NCC-Spd to NCC-Put presented in Table 1, we found that exogenous Spd treatment elevated the ratio in PEG-treated Yangmai No. 9 cv., but it affected the ratio in PEG-treated Yumai No. 18 cv. to a lesser extent. MGBG treatment reduced the ratio in PEG-treated Yumai No. 18 cv. more significantly (Table 1).

Table 1. Effects of PEG, PEG+Spd and PEG+MGBG on the Levels of Noncovalently Conjugated PAs (NCC-PAs) in Tonoplast Vesicles Isolated from Wheat Seedling Roots

Cultivars	Treatments	NCC-PA levels		
		Put nmol·mg^{-1} protein	Spd	Spd/Put
Yangmai No. 9	Control	0.41 ± 0.08 e	0.71 ± 0.15 e	1.73
	PEG	1.30 ± 0.16 b	0.91 ± 0.17d	0.70
	PEG+Spd	1.44 ± 0.20 a	1.98 ± 0.27 a	1.31
	PEG+MGBG	1.46 ± 0.20 a	0.89 ± 0.15 d	0.61
Yumai No.18	Control	0.35 ± 0.07 e	0.65 ± 0.13 e	1.86
	PEG	0.86 ± 0.09 d	1.33 ± 0.21 c	1.55
	PEG+Spd	1.08 ± 0.10 c	1.72 ± 0.24 b	1.59
	PEG+MGBG	0.94 ± 0.10 d	0.98 ± 0.16 d	1.04

Wheat seedlings were treated with PEG (−0.55 MPa), PEG (−0.55 MPa)+Spd (1 mM), or PEG (−0.55 MPa)+MGBG (1 mM) for 7 d. The roots were then sampled for isolation of tonoplast vesicles. NCC-PA levels were determined by HPLC. Means with different letters (a ∼ e) within a column are significantly different at $p < 0.05$ based on Duncan's multiple range test. The values are the mean +SE (n = 9) of three experiments.

The Direct Effect of Spd on H$^+$-ATPase and H$^+$-PPase Activities of Tonoplast Vesicles Isolated from Wheat Seedlings

Exogenous Spd increased the H$^+$-ATPase (Figure 3A) and H$^+$-PPase (Figure 3B) activities of tonoplast vesicles isolated from controls of both wheat cvs., implying that exogenous Spd might modulate H$^+$-ATPase and H$^+$-PPase via binding noncovalently to proteins of tonoplast vesicles.

Effects of PEG and o-Phen on Level of Covalently Conjugated Polyamines of Root Tonoplast Vesicles from Wheat Seedlings

The levels of the two CC-PAs (CC-Put and CC-Spd) could be detected in root tonoplast vesicles from PEG-treated seedlings of the two wheat cultivars, but the CC-Spm level could not be detected. Under PEG-induced osmotic stress, the CC-Put level in Yumai No. 18 cv. seedlings increased much more than that in Yangmai No. 9 seedlings. However, there was no significant difference in the CC-Spd level between the two wheat cvs. (data not shown). Exogenous o-Phen treatment inhibited the PEG-induced increase of CC-Put levels in root tonoplast vesicles of Yumai No. 18 cv. seedlings more significantly than it did in Yangmai No. 9 cv. (Table 2).

The Relationship Between Activities of H$^+$-ATPase and H$^+$-PPase and the Levels of Noncovalently Conjugated and Covalently Conjugated Polyamines

To elucidate further the relation between PAs and H$^+$-ATPase and H$^+$-PPase, we analyzed the ratio of NCC-Spd level to NCC-Put level, CC-Put level and the activities of H$^+$-ATPase and H$^+$-PPase in root tonoplast vesicles. Results showed that there was a significant positive correlation between the ratio NCC-Spd/NCC-Put and H$^+$-ATPase activity ($r = 0.97$, $r_{0.01} = 0.83$, $n = 8$) (Figure 4a) and between the ratio NCC-Spd/NCC-Put and H$^+$-PPase activity ($r = 0.96$, $r_{0.01} = 0.83$, $n = 8$) (Figure 4b). Statistical analysis indicated that under osmotic stress, the CC-Put level was positively related to H$^+$-ATPase activity ($r = 0.91$, $r_{0.1} = 0.90$, $n = 4$) (Figure 4c) and H$^+$-PPase activity ($r = 0.94$, $r_{0.1} = 0.90$, $n = 4$) (Figure 4d) in root tonoplast vesicles of wheat seedlings.

Discussion

Effect of Osmotic Stress on the Activities of H$^+$-ATPase and H$^+$-PPase in Root Tonoplast Vesicles from Wheat Seedlings

Plant tolerance to water stress has been associated with higher LRWC and RDWIR of plants subjected to stress (Hsiao 1973; Schonfeld and others 1988). Thus, from the results in Figure 1, we confirmed that cv. Yumai No. 18 is osmotic tolerant and cv. Yangmai No. 9 is osmotic sensitive.

PEG treatment decreased the H$^+$-ATPase and H$^+$-PPase activities in root tonoplast vesicles isolated from seedlings of the drought-sensitive Yangmai No. 9 cv. much more markedly than those from the drought-tolerant Yumai No. 18 cv. (Figure 1). This result indicated that the H$^+$-ATPase and H$^+$-PPase were possibly involved in the water stress tolerance

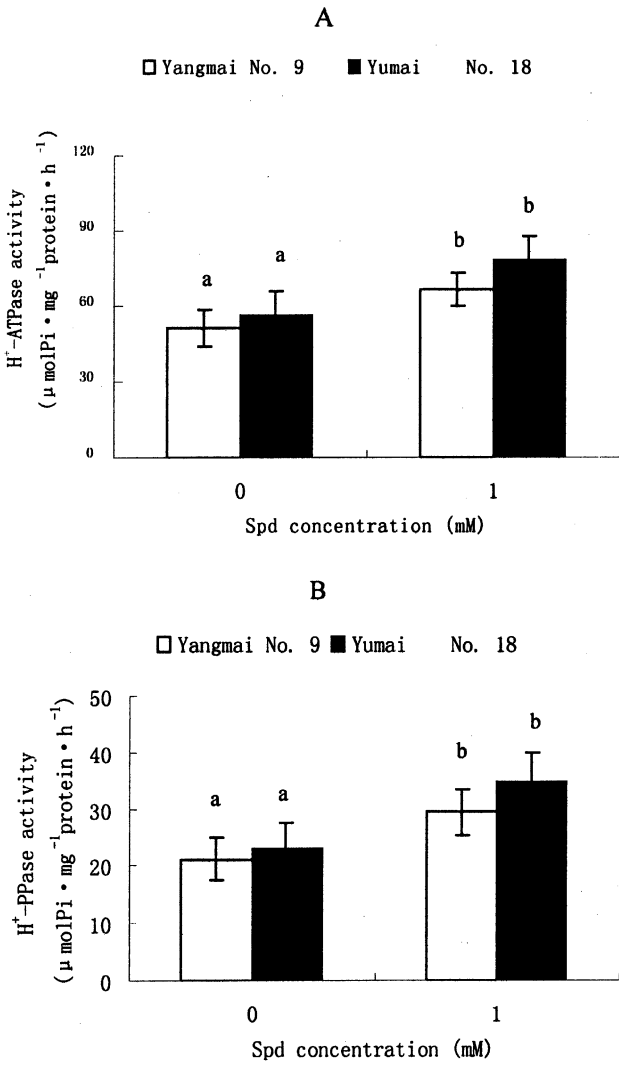

Figure 3. The direct effect of Spd on the activities of H^+-ATPase (**A**) and H^+-PPase (**B**) of isolated tonoplast vesicles, *in vitro*. Root tonoplast vesicles isolated from control wheat seedlings were pre-incubated in the medium for H^+-ATPase (or H^+-PPase) assay into which Spd (1 mM) was added at 37°C for 10 min before adding ATP (or PPi) to start the reaction. Error bars indicate SE (n = 9). Means with different letters (a, b) are significantly different at $p < 0.05$ based on Duncan's univariate range test.

of wheat seedlings. Two further studies supported this hypothesis: first, exogenous Spd treatment alleviated PEG osmotic stress injury to Yangmai No. 9 cv. seedlings (Figure 1) leading to significant inhibition of the PEG-induced decrease of H^+-ATPase and H^+-PPase activities in tonoplast vesicles of seedling roots (Fig. 2); second, exogenous MGBG or o-Phen treatment aggravated the PEG-induced injury to Yumai No. 18 cv. seedlings (Figure 1), with marked enhancement of the PEG-induced decrease of H^+-ATPase and H^+-PPase activities (Figure 2) in

Table 2. Effects of PEG and PEG+o-Phen on the Levels of Covalently Conjugated PAs (CC-PAs) in Tonoplast Vesicles Isolated from Wheat Seedling Roots

Cultivars	Treatments	CC-PA Put level
		(nmol·mg^{-1} protein)
Yangmai No. 9	Control	0.31 ± 0.02 d
	PEG	0.38 ± 0.04 bc
	PEG+o-Phen	0.35 ± 0.04 cd
Yumai No. 18	Control	0.33 ± 0.03 c
	PEG	0.59 ± 0.09 a
	PEG+o-Phen	0.43 ± 0.05

Wheat seedlings were treated with PEG (−0.55 MPa) or PEG (−0.55 MPa)+o-Phen (1 mM) for 7 d, respectively. The roots were then sampled for isolation of tonoplast vesicles. CC-PA levels were determined by HPLC. Means with different letters (a ~ c) within a column are significantly different at $p < 0.05$ based on Duncan's multiple range test. The values are the mean ± SE (n = 9) of three experiments.

tonoplast vesicles of seedling roots. Our finding is consistent with the results of Shantha and others (2001) and Wang and others (2001). Although the research of Wang and others (2001) showed that H^+-ATPase activity in tonoplast vesicle fractions from PEG-treated *Suaeda salsa* plant was not significantly different from values obtained for controls, the data showed that osmotic stress markedly decreased H^+-PPase activity 3-fold after 8 d treatment. Because water flow into the expanding vacuole is driven by ion accumulation, which in turn is energized by the vacuolar H^+-ATPase (Viereck and others 1996) and because maintenance of vacuolar compartmentation is of fundamental importance, particularly under conditions of water and turgor loss, the activity of the vacuolar H^+-ATPase might be correlated to the degree of osmotic stress. There are reports on the lack of effect of osmotic stress on tonoplast proton pump activity (Colombo and Cerana 1993; Vera-Estrella and others 1999).

Relationship Between the Activities of H^+-ATPase and H^+-PPase and Levels of Noncovalently Conjugated Polyamines in Tonoplast Vesicles from Wheat Seedling Roots

Lester (2000) reported the effects of exogenous PAs on the activities of membrane-associated enzymes. Our previous work (Liu and others 2004) showed that free Spd and free Spm facilitated osmotic stress tolerance of wheat seedlings. However, there are few investigations of the relationship between membrane-associated PA levels and enzyme activities. Here, we detected noncovalently conjugated

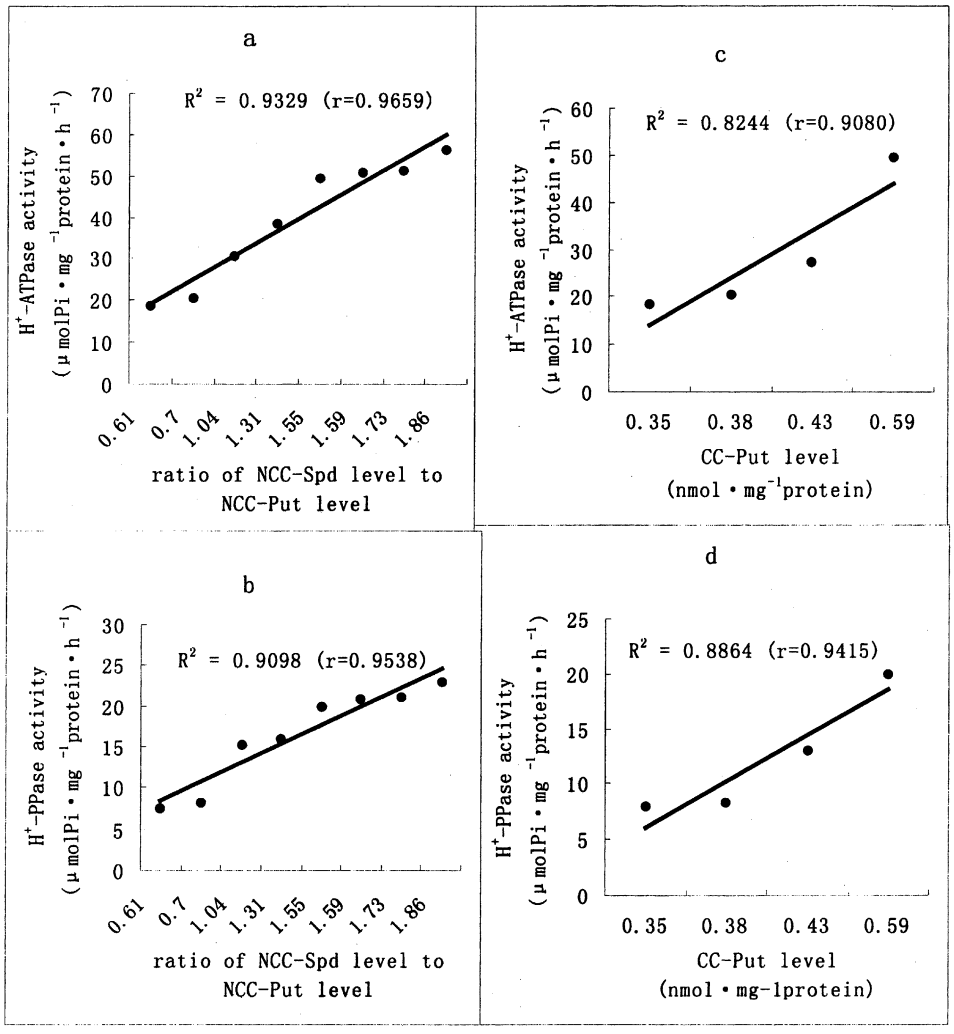

Figure 4. The relationship between the activities of H^+-ATPase and H^+-PPase and the levels of NCC-PAs and CC-PAs. (**a**) Relationship between the ratio of NCC-Spd level to NCC-Put level and H^+-ATPase activity in root tonoplast vesicles from wheat seedlings. (**b**) Relationship between the ratio of NCC-Spd level to NCC-Put level and H^+-PPase activity in root tonoplast vesicles from wheat seedlings. (**c**) Relationship between CC-Put level and H^+-ATPase activity in root tonoplast vesicles from wheat seedlings. (**d**) Relationship between CC-Put level and H^+-PPase activity in root tonoplast vesicles from wheat seedlings.

PAs (NCC-PAs) in tonoplast vesicles. The results showed that PEG treatment caused much more significant increases of tonoplast NCC-Spd levels in tonoplasts derived from the drought-tolerant Yumai No. 18 cv. than those from the drought-sensitive Yangmai No. 9 cv. (Table 1). These findings, together with the result that PEG-treated Yumai No. 18 seedlings could maintain higher H^+-ATPase and H^+-PPase activities in root tonoplast vesicles than Yangmai No. 9 seedlings (Figure 2), were indicative of possible involvement of NCC-Spd in the maintenance of the activity of these two enzymes under osmotic stress. This hypothesis was supported by the following observations: first, PEG-induced increases in tonoplast NCC-Spd levels in Yumai No. 18 cv. seedling roots was inhibited by MGBG treatment (Table 1), with enhancement of PEG-induced decreases of H^+-ATPase and H^+-PPase activities of this cultivar (Figure 2); second, exogenous Spd treatment markedly increased tonoplast NCC-Spd levels (Table 1) and inhibited PEG-induced decreases of tonoplast H^+-ATPase and H^+-PPase activities in Yangmai No. 9 seedling roots (Figure 2). Statistical analysis also indicated that there was significant positive correlation between the ratio NCC-Spd/NCC-Put and H^+-ATPase activity ($r = 0.97$, $r_{0.01} = 0.83$, $n = 8$) (Figure 4a) and H^+-PPase activity ($r = 0.96$, $r_{0.01.} = 0.83$, $n = 8$)(Figure 4b). Exogenous Spd increased the H^+-ATPase (Figure 3A) and H^+-PPase (Figure 3B) activities of tonoplast vesicles isolated from control seedlings of both wheat cv., implying that exogenous Spd might modulate directly H^+-ATPase and H^+-PPase via binding noncovalently to proteins of the tonoplast vesicles.

Our finding was consistent with the observation that the ratio of (NCC-Spd + NCC-PAx (an unknown PA) to (NCC-Put + NCC-diaminopropane) was significantly positively correlated with the activities of the enzymes H^+-ATPase and H^+-PPase

in the tonoplast of barley seedlings under salt stress (Sun and others 2002). One reason why NCC-Spd could stimulate the H^+-ATPase and H^+-PPase activities was attributed to its poly-cationic nature at physiological pH. Thus, Spd affects the physical state of the tonoplast by noncovalently binding to the negative charges of membrane phospholipids. It may also affect protein conformation and function by noncovalently binding to negative charges of acidic membrane proteins (including the tonpoplast H^+-ATPase and H^+-PPase) more easily. However, the study of Dobrovinskaya and others (1999) showed that vacuolar ion channels were inhibited by PAs and the findings of Liu and others (2000) suggest that polyamines inhibited KAT_1-like channels in guard cells.

Taking our work together with others, we can infer that because there are many kinds of membrane-associated proteins in the tonoplast, such as pumps, carriers and channels, NCC-PAs in the tonoplast may modulate these proteins via a range of different mechanisms. Identifying these proteins and their mode of interaction with PAs deserves further investigation.

Relationship Between the Activities of H^+-ATPase and H^+-PPase and Levels of Covalently Conjugated Polyamines in Root Tonoplast Vesicles from Wheat Seedlings

In the present research, we also detected the covalently conjugated polyamines (CC-PAs) in tonoplast vesicles from PEG-treated wheat seedling roots. The result in Table 2 showed that PEG treatment increased CC-Put levels in drought-tolerant Yumai No. 18 cv. more markedly than that in drought-sensitive Yangmai No. 9 cv. seedlings. These results, together with the results on H^+-ATPase and H^+-PPase activities (Figure 2), suggested that H^+-ATPase and H^+-PPase activities were associated with the level of CC-Put, whereas CC-Spd has no relationship with the activities of the two enzymes because of its minor level under osmotic stress (data not shown). This suggestion was supported by the effect of concomitant treatment with o-Phen, an inhibitor of TGase, which enhanced the PEG-induced decrease of H^+-ATPase and H^+-PPase activities (Figure 2), coupled with inhibition of CC-Put biosynthesis in the tonoplast (Table 2). This idea was also supported by the positive correlation between CC-Put levels and H^+-ATPase activity ($r = 0.91$, $r_{0.1} = 0.90$, $n = 4$) (Figure 4: c) and H^+-PPase activity ($r = 0.94$, $r_{0.1} = 0.90$, $n = 4$) (Figure 4: d) in root tonoplasts of wheat seedlings subjected to osmotic stress. Del Duca and others (1995) suggested that polyamines might have an important function in chloroplasts both in their free form and by covalently binding to proteins. An extensive literature has emphasized the involvement of PAs in post-translational modification of proteins in the reaction catalyzed by TGases. Del Duca and others (1995) also provided evidence that PAs are covalently bound to endogiutamyl residues of the chlorophyll *a/b* antenna complex, CP26, CP24, CP26 and the large subunit of Rubisco. It was hypothesized that Put might mediate membrane wounding (DiTomaso and others 1989), so the conversion of free Put to CC-Put could alleviate the free Put wounding effect. Furthermore, the conversion of free Put to CC-Put, forming protein-Gln-Put and protein-Gln-Put-protein, could stabilize the configuration and function of tonoplast proteins (including the H^+-ATPase and H^+-PPase) by preventing the proteins from denaturing under PEG-induced osmotic stress, and so protect enzyme activity (Serafini-Fracassini 1995).

In summary, to our knowledge, the present report is the first to demonstrate that NCC-Spd and CC-Put were involved in the maintenance of the H^+-ATPase and H^+-PPase activities in tonoplast vesicles isolated from seedling roots under osmotic stress, and that they may be involved in osmotic stress tolerance of wheat seedlings. Thus, this investigation may help better the understanding of the role of various forms of PAs in osmotic stress tolerance of plants.

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