Plant Growth Regul (2015) 75:1–10
DOI 10.1007/s10725-014-9925-9

ORIGINAL PAPER

Changes in H^+-ATPase activity and conjugated polyamine contents in plasma membrane purified from developing wheat embryos under short-time drought stress

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Received: 25 December 2013 / Accepted: 12 April 2014 / Published online: 20 April 2014

Abstract Plasma membrane (PM) vesicles were isolated from developing embryos of wheat (*Triticum aestivum* L.) with different drought-tolerance under drought stress by the gradient centrifugation method. The activity of the PM H^+-ATPase (EC 3.6.1.35) and contents of polyamine conjugated (covalently and noncovalently) to the PM vesicles were investigated. Results showed that after drought treatment for 3 d, embryo relative water content (ERWC), embryo relative dry weight increase rate (ERDWIR) of drought-sensitive Yumai No. 48 cultivar decreased more significantly than those of drought-tolerant Luomai No. 22 cultivar, while PM H^+-ATPase activity, noncovalently conjugated (NCC) spermidine (Spd) and NCC spermine (Spm), the covalently conjugated (CC) putrescine (Put) and CC Spd of PM from Luomai No. 22 cultivar increased more obviously than those from Yumai No. 48 cultivar. As judged by increases in ERWC and ERDWIR, treatment with exogenous Spd alleviated markedly drought injuries to Yumai No. 48, coupled with significant increases in NCC Spd and NCC Spm levels and H^+-ATPase activity in the embryo PM vesicle. Under drought stress, the treatment of drought-tolerant Luomai No. 22 cultivar with methylglyoxyl-bis (guanylhydrazone) (MGBG), an inhibitor of *S*-adenosylmethionine decarboxylase (SAMDC), and phenanthrolin (o^-Phen), an inhibitor of transglutaminase (TGase) respectively, caused a decrease of the NCC Spd, NCC Spm, CC Put and CC Spd. Those decreases were associated with decreased PM-H^+-ATPase activity and the tolerance of developing wheat embryos to osmotic stress, as judged by decreases in ERWC and ERDWIR. These results suggest that tolerance of the developing wheat embryos to drought stress is associated with the embryo PM H^+-ATPase and the levels of NCC Spd, NCC Spm, CC Put and CC Spd in embryo PM vesicles.

Keywords Drought stress · Conjugated polyamines · Plasma membrane H^+-ATPase · Wheat (*Triticum aestivum* L.) embryo

Abbreviations

BSA	Bovine serum albumin
CC	Covalently conjugated
DR	Drought
DTT	Dithiothreitol
EGTA	Ethylene glycol tetraacetic acid
ERDWIR	Embryo relative dry weight increase rate
ERWC	Embryo relative water content
Hepes	2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
HPLC	High performance liquid chromatography
MGBG	Methylglyoxyl-bis (guanylhydrazone)
NCC	Noncovalently conjugated
O^-Phen	Phenanthrolin
PA	Polyamine
PCA	Perchloric acid
PEG	Polyethylene glycol–6000

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PM	Plasma membrane
PMSF	Phenylmethanesulfonyl fluoride
Put	Putrescine
PVP	Polyvinyl pyrrolidone
SAMDC	*S*-adenosylmethionine decarboxylase
Spd	Spermidine
Spm	Spermine
TCA	Trichloroacetic acid
TGase	Transglutaminase

Introduction

Polyamines (PAs) are biologically aliphatic amines. In plants, PAs include mainly putrescine (Put), spermidine (Spd) and spermine (Spm). The synthesis of Spd and Spm is catalyzed by Spd synthase and Spm synthase via incorporation of aminopropyl to Put and Spd, respectively. S-adenosylmethionine decarboxylase (SAMDC) catalyzes the transformation of S-adenosylmethionine to decarboxylated S-adenosylmethionine, a donor of the aminopropyl to Put and Spd. The Spd and Spm biosynthesis is mainly regulated by SAMDC (Tiburcio et al. 1993). Methylglyoxal-bis (guanylhydrazone) (MGBG) is the potent inhibitor of SAMDC (Slocum 1991). PAs are involved in many aspects of growing and development (Adiga and Prasad 1985; Martin-Tanguy 2001; Iqbal and Ashraf 2005; Cao et al. 2010; Niemenak et al. 2012; Cacho et al. 2013). Furthermore, many studies show that PAs are closely associated with abiotic stress such as osmotic, drought, salt and heavy metal stress, etc. (Tang and Newton 2005; Liu et al. 2006; Goyal and Asthir 2010; Qiao et al. 2012; Do et al. 2013; Grzesiak et al. 2013). Due to their positive charges at physiological pH, PAs are able to bind to macromolecules such as DNA, RNA, and proteins by hydrogen and ionic bonding (Sood and Nagar 2003). By being conjugated to proteins in the Plasma membrane (PM), they could be transformed into noncovalently conjugated PAs (NCC PAs), which are considered to play an important role in stabilizing the function of protein and biomembrane for stress adaptation (Galston and Kaur-Sawhney 1995). Besides the two form PAs mentioned above, by action of Transglutaminase (TGase, E.C. 2.3.2.13), which covalently links free PAs to endoglutamines of proteins, PAs could be transformed into covalently conjugated PAs (CC PAs). Phenanthrolin (o^{-}Phen) is a potent inhibitor of TGase. CC PAs play important roles in the process of post-translational modifications of proteins (Del Duca et al. 1995) and facilitate wheat seedling tolerance to osmotic stress (Liu et al. 2004).

The plant PM H^+-ATPase (E.C. 3.6.1.35), P-type ATPase, is a proton pump with ATP hydrolyzing, which plays a key role in plant growth and development and controls lots of cellular processes, such as secondary active transport, which is correlated with cell pH, turgor, and stress adaption (Surowy and Boyer 1991; Sailerova and Zwiazek 1993; Michelet et al. 1994; Michelet and Boutry 1995). Structural analysis indicates that the *C*-terminus of the P-type H^+-ATPase is an auto-inhibitory domain, which is removed with the enzyme to be activated (Palmgren et al. 1991; Johansson et al. 1993). The P-type H^+-ATPase is regulated at various levels such as the pre-transcriptional, transcriptional, and post-transcriptional levels (Sussman and Harper 1989; Sussman 1994) and modulated by calcium ions, abscisic acid, auxin, cGMP, light, phospholipids, kinases, and various environmental factors, such as drought, cold, salt, etc. (Serrano 1989; Kinoshita et al. 1995; Suwastika and Gehring 1999; Peng et al. 2003). Therefore, PM H^+-ATPase is thought to be one of the important enzymes which are involved in plant response to environmental stresses. Under drought stress, the reports on changes in PM- H^+-ATPase activity in plants are inconsistent. Some research indicates that PM H^+-ATPase activity is enhanced (Michelet et al. 1994), while other reports demonstrate that its activity is inhibited (Qiu and Zhang 2000).

Drought is one of the main environmental stresses which influence crop growth and development and wheat seed is frequently subjected to drought stress during development. However, to our knowledge, the relationship between PM H^+-ATPase activity and levels of noncovalently conjugated (NCC) and CC PAs in PM, purified from developing wheat embryos under drought stress, remains to be elucidated. So, in the experiments presented here, two wheat (*Triticum aestivum* L.) cultivars (drought-sensitive Yumai No. 48 cv. and drought-tolerant Luomai No. 22 cv.) were used as materials, and we detected PM- H^+-ATPase activity and the two conjugated PAs (NCC and CC PAs) in PM vesicles purified from developing wheat embryos subjected to drought stress. The main aim of this work is to elucidate the significances of NCC PAs and CC PAs in PM of wheat embryos under drought stress. A possible relationship between the activity of PM H^+-ATPase and the function of the two forms of conjugated PAs is discussed.

Materials and methods

Material and treatments

Wheat (*Triticum aestivum* L. cv. Luomai No. 22, drought-tolerant; cv. Yumai No. 48, drought-sensitive) seed surface was sterilized in 0.1 % $HgCl_2$ (w/v) for 5 min before they were rinsed with water, and then germinated in pots (30 seeds/pot) (bottom diameter:rim diameter: height: 35: 40: 50 cm) containing nutrient-rich and water–normal topsoil. After the wheat seedlings were vernalized, the pots with

seedlings were placed in an environment greenhouse with a temperature of 25/15 °C (day/night), air humidity of 70 %, and 16 h photoperiod at quantum flux density of 600 μmol m^{-2} s^{-1} from lamps. On the 10th day after fertilization, the growing wheat plants were treated as followings: (1) Wheat roots were treated under −1.0 MPa soil drought stress; (2) Wheat roots were treated under −1.0 MPa soil drought stress and the wheat flag leaves and ears were sprayed with Spd (1 mM); (3) Wheat roots were treated under −1.0 MPa soil drought stress and the wheat flag leaves and ears were sprayed with MGBG (1 mM); (4) Wheat roots were treated under −1.0 MPa soil drought stress and the wheat flag leaves and ears were sprayed with o^{-}Phen (1 mM). Spd, MGBG, and o^{-}Phen were purchased from Sigma Chemical Co. The materials, of which wheat roots were treated with water–normal soil (−0.15 MPa) and the wheat flag leaves and ears were sprayed with distilled water, were as control group materials. Soil water potential was detected by TEN60 Water Potential Instrument. Wheat flag leaves and ears were sprayed with the reagents mentioned above by 20 ml/pot at 6:00 and 18:00 h per day. After treatment for 3 d, the developing seed embryos were sampled and tested.

Determination of embryo relative water content (ERWC)

Embryo relative water content (ERWC) was calculated according to the following formula: ERWC (%) = $(W_f - W_d)/(W_t - W_d) \times 100$ (W_f, W_d, and W_t represents the embryo fresh weight, dry weight and saturation weight, respectively).

Determination of embryo relative dry weight increase rate (ERDWIR)

Growth rate of embryo dry weight was calculated by the following formula: GR = $(W_a - W_b)/W_b$ (W_a and W_b represents the dry weight of the embryos after 3 and 0 d of treatments, respectively). And then, to counteract the diversity of different cultivar, embryo relative dry weight increase rate (ERDWIR) of every cultivar with different treatment was calculated by the following formula: ERDWIR (%) = (GR of treatment/GR of control) × 100 (Where treatment and control were the same cultivar).

Preparation of embryo PM vesicles and H^+-ATPase activity determination

Embryo PM vesicles were purified by sucrose gradient centrifugation methods according to the process described by Qiu and Su (1998) with slight modifications as follows. 2 g fresh embryos were cut into pieces and homogenized in 10 ml of ice-cold extracting solution containing 100 mM KCl, 200 mM sorbitol, 4 mM EGTA, 1 mM PMSF, 2 mM DTT, 15 % (v/v) glycerol, 1.5 % (w/v) PVP, 0.2 % (w/v) BSA, 200 mM $K_2S_2O_5$ and 60 mM Tris-Hepes (pH 7.8). The homogenate was filtered through 4 layers of gauze and centrifugated at 12,000×*g* for 20 min. The supernatant was centrifugated for 35 min at 80,000×*g* to get microsomal membrane precipitate, which was slightly suspended in a buffer containing 0.2 M sucrose, 15 mM KCl, 2 mM DTT, 2 mM EGTA, 0.2 % (w/v) BSA and 2.5 mM Hepes titrated to pH 7.5 with Tris.

The suspending microsomal membrane was layered in discontinuous sucrose gradient solutions, which consisted of 34 and 41 % (w/w) sucrose layers, containing 2 mM DTT and 2.5 mM Hepes titrated to pH 7.5 with Tris. Then the mixture was centrifugated at 120,000×*g* for 100 min. The fraction was collected at the interface of 34–41 % sucrose solution and diluted fourfold with the suspension buffer mentioned above. Then the suspension was centrifugated at 75,000×*g* for 35 min. The PM pellet was then collected. The purity of the PM vesicles was estimated following the method described by Widell and Larsson (1990). The PM, tonoplast and mitochondria H^+-ATPase are characterized by vanadate, nitrate and azide inhibition, respectively. In the present research, the H^+-ATPase activity of prepared PM vesicle was inhibited by vanadate more than 80 %, and inhibited by nitrate and azide <2 and 1.5 %, respectively, which demonstrated that the purity of isolated PM vesicle was very high.

H^+-ATPase activity was assayed by measuring inorganic phosphate from ATP following the method described by Qiu and Su (1998) and Ohinishi et al. (1975) with minor modification. The reaction solution contained 4 mM ATP-Na_2, 2.5 mM $MgSO_4$, 0.2 mM Na_2MoO_4, 1.5 mM NaN_3, 60 mM KNO_3, 30 mM Hepes titrated to pH 6.5 with Tris and 8 μg proteins membrane vesicles. After 35 min incubation at 37 °C, 15 % (w/v) TCA quenched the reaction.

Determination of PM proteins

To isolate the protein, the prepared PM vesicle solution was added to Triton X-100 1 % (v/v). The solution was then supersonic-treated three times for 20 s with an ultrasonic disintegrator (model 200-w), kept in an ice bath for 35 min, and centrifugated at 22,000×*g* for 25 min at 4 °C (Zhao et al. 2000). The content of the soluble membrane protein in the supernatant was determined with the method described by Bradford (1976), BSA being standard.

Determination of conjugated PA

Conjugated PAs were quantified following the method described by Sharma and Rajam (1995) with minor modification. PCA was dropwise added to the membrane protein extract mentioned above to 5 % terminal concentration, and

then the solution was centrifugated at 30,000×*g* for 45 min. The precipitate was re-suspended in 5 % PCA, mixed with equal volume of 12 N HCl in ampoule, hydrolyzed at 110 °C for 24 h after the ampoule was sealed, and dried at 70 °C after filtration. The pellet was re-dissolved in 2 ml 5 % PCA. The solution contained the CC PAs.

After 2 ml 5 % PCA was added to the purified membrane vesicles, it was centrifugated at 28,000×*g* for 35 min. The supernatant contained the NCC PAs. The CC and NCC PAs in two solutions above were derivatized with benzoyl chloride respectively following the method described by Di Tomaso et al. (1989) and quantified by HPLC (Waters 2695 with automatic sample injector), with 254 nm as PA detecting wavelength, C-18 reverse-phase column as separation column and 1,6-hexanediamine as an internal standard.

Cultivation and treatment of young wheat embryos, in vitro

To verify the aforementioned experiments, in vivo, the following experiments, in vitro, were carried out. On the 10th day after fertilization, young wheat embryos were taken out from developing seed, and inoculated onto sterile MS solid medium. After cultivation for 3 d, the embryos were treated with 20 % PEG-6000 (−0.5 MPa), 20 % PEG (−0.5 MPa) + Spd (0.5 mM), 20 % PEG (−0.5 MPa) + MGBG (0.5 mM) and 20 % PEG (−0.5 MPa) + o^{-}Phen (0.5 mM) for 2 d, respectively and control embryos were kept on sterile MS solid medium without PEG, Spd, MGBG or o^{-}Phen. Then, the treated and control embryos were sampled for assays of H^+-ATPase activity and conjugated PAs in PM following the methods mentioned above.

Statistical analysis

The experiment was repeated three times and 3 samples were taken every experiment. Data were analysed using software of SPSS 10.0 and Microsoft Excel. Values reported in the paper are means ± stand error (SE) of three independent tests. Significant differences among the samples were determined by Duncan's multiple range tests at a significance level of 0.05.

Results

Effects of drought stress, exogenous Spd, MGBG and o^{-}Phen on ERWC and ERDWIR of developing wheat embryos

Resistance to drought stress has been associated with the higher ERWC and ERDWIR of stressed plants (Schonfeld et al. 1988). Drought stress for 3 d caused decreases of ERWC and ERDWIR of two wheat cultivars, Yumai No. 48 and Luomai No. 22, and the changes in the former cultivar were more apparent than those in the latter (Fig. 1). This result proved that Luomai No. 22 was drought-tolerant and Yumai No. 48 was drought-sensitive. Treatment of Yumai No. 48 with exogenous Spd alleviated the drought injury, as judged by increases in ERWC and ERDWIR, while exogenous Spd affected the resistance of Luomai No. 22 slightly. Drought injury to Luomai No. 22 was greater compared to that of Yumai No. 48 after treatment with MGBG, as judged by the larger decreased ranges of ERWC and ERDWIR (Fig. 1). O^{-}Phen treatment enhanced the drought-induced decreases of ERWC and EDWIR of both cultivars, and the effect on Yumai No. 48 was slighter than that on Luomai No. 22 (Fig. 1).

To elucidate the mechanism of presentation above, the following tests were presented.

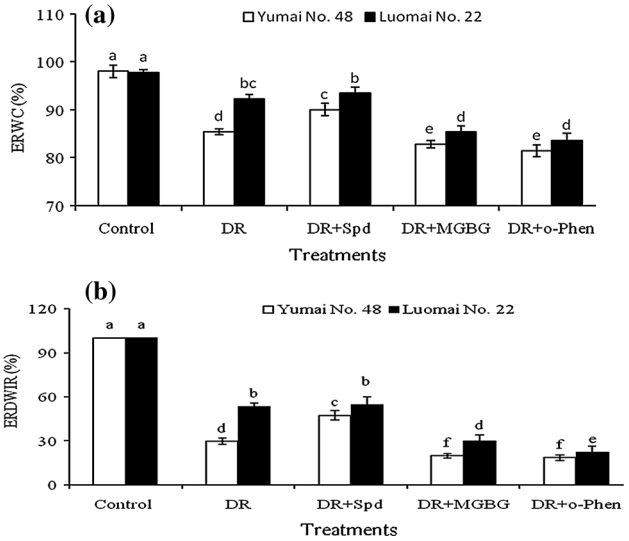

Fig. 1 Effects of drought stress, exogenous Spd, MGBG and o^{-}Phen on ERWC (**a**) and ERDWIR (**b**) of developing wheat embryos. On the 10th day after fertilization, the wheat roots were treated under −1.0 MPa soil drought stress and the wheat roots with water–normal soil were as control group. The wheat flag leaves and ears were sprayed with Spd (1 mM), MGBG (1 mM) and o^{-}Phen (1 mM), respectively. The control group was sprayed with distilled water. After treatment for 3 d, the developing seed embryos were sampled and tested. Each value in the figure represents the mean of three experiments ± SE (standard error), *error bars* indicate SE (n = 9), and *different letters* (*a–f*) above the column are significantly different at $P < 0.05$ (Duncan's multiple range tests). DR, drought stress treatment; DR + Spd, treatment with drought stress and Spd (by spraying 1 mM) simultaneously; DR + MGBG, treatment with drought stress and MGBG (by spraying 1 mM) simultaneously; DR + o^{-}Phen, treatment with drought stress and o^{-}Phen (by spraying 1 mM) simultaneously

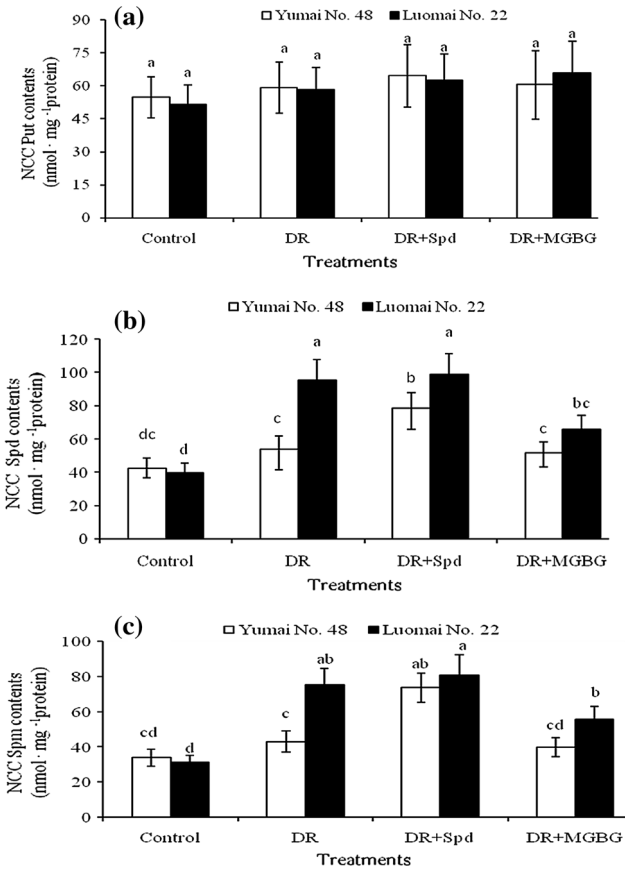

Fig. 2 Effects of drought stress, exogenous Spd, MGBG on NCC Put (**a**), NCC Spd (**b**) and NCC Spm (**c**) contents in PM vesicles from developing wheat embryos. On the 10th day after fertilization, the wheat roots were treated under −1.0 MPa soil drought stress and the wheat roots with water–normal soil were as control group. The wheat flag leaves and ears were sprayed with Spd (1 mM) and MGBG (1 mM), respectively. The control group was sprayed with distilled water. After treatment for 3 d, the developing seed embryos were sampled and tested. Each value in the figure represents the mean of three experiments ± SE (standard error), *error bars* indicate SE (n = 9), and *different letters* (*a–d*) above the column are significantly different at $P < 0.05$ (Duncan's multiple range tests). DR, drought stress treatment; DR + Spd, treatment with drought stress and Spd (by spraying 1 mM) simultaneously; DR + MGBG, treatment with drought stress and MGBG (by spraying 1 mM) simultaneously

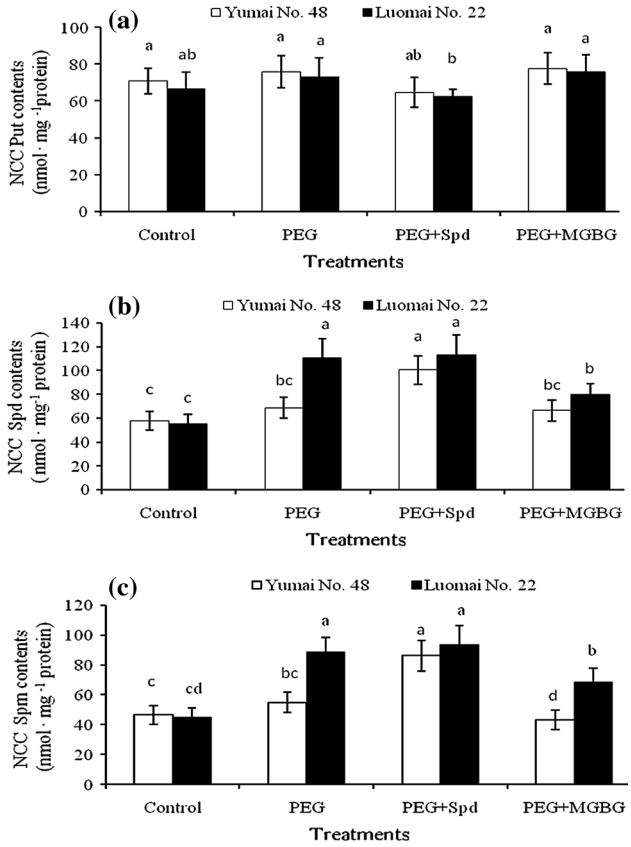

Fig. 3 Effects of PEG, exogenous Spd, MGBG on NCC Put (**a**), NCC Spd (**b**) and NCC Spm (**c**) contents in PM vesicles from wheat embryos developing on MS solid medium, in vitro. On the 10th day after fertilization, young wheat embryos were taken out from developing seed and inoculated onto MS solid medium. After cultivation for 3 d, the embryos were treated with PEG (−0.5 MPa), PEG (−0.5 MPa) + Spd (0.5 mM), and PEG (−0.5 MPa) + MGBG (0.5 mM) for 2 d, respectively and control embryos were kept on MS solid medium without PEG, Spd or MGBG. Then, the treated and control embryos were sampled for assays of NCC PAs in PM. Each value in the figure represents the mean of three experiments ± SE (standard error), *error bars* indicate SE (n = 9), and *different letters* (*a–d*) above the column are significantly different at $P < 0.05$ (Duncan's multiple range tests). PEG, treatment with PEG; PEG + Spd, treatment with PEG and Spd simultaneously; PEG + MGBG, treatment with PEG and MGBG simultaneously

Effects of drought stress, exogenous Spd, MGBG on NCC PA contents in PM vesicles from developing wheat embryos

Drought stress brought about increases in contents of NCC Put, NCC Spd and NCC Spm in PM vesicles purified from both wheat cultivar embryos (Fig. 2a, b, c), and the contents of NCC Spd and NCC Spm of Luomai No. 22 increased more markedly than those of Yumai No. 48 (Fig. 2b, c). Exogenous Spd treatment caused a significant increase in the contents of NCC Spd and NCC Spm in PM vesicles from Yumai No. 48 embryos under drought stress and the effects of Spd on Luomai No. 22 were slight (Fig. 2b, c). MGBG treatment caused a significant reduction in the contents of NCC Spd and NCC Spm in PM vesicles from the developing embryos of Luomai No. 22 under drought stress and the effect of MGBG on Yumai No. 48 was slight (Fig. 2b, c).

Effects of PEG, exogenous Spd, MGBG on NCC PA contents in PM vesicles from wheat embryos developing on MS solid medium, in vitro

To elucidate further the changes in NCC PAs from PM vesicles, we used wheat embryos developing on the MS

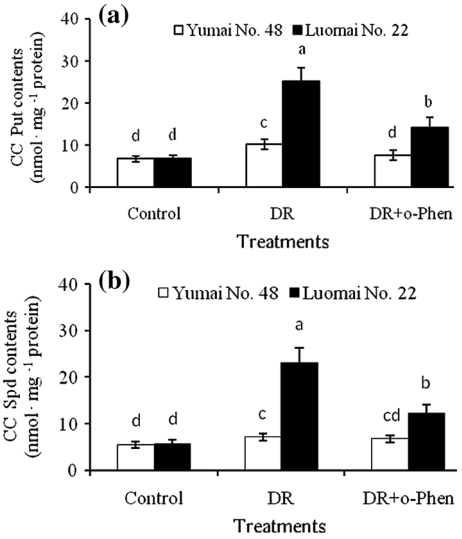

Fig. 4 Effects of drought stress and o^{-}Phen on CC Put and CC Spd contents in PM vesicles from developing wheat embryo. On the 10th day after fertilization, the wheat roots were treated under −1.0 MPa soil drought stress and the wheat roots with water–normal soil were as control group. The wheat flag leaves and ears were sprayed with o^{-}Phen (1 mM). The control was sprayed with distilled water. After treatment for 3 d, the developing seed embryos were sampled and tested. Each value in the figure represents the mean of three experiments ±SE (standard error), *error bars* indicate SE (n = 9), and *different letters* (*a–d*) above the column are significantly different at $P < 0.05$ (Duncan's multiple range tests). DR, drought stress treatment; DR + o^{-}Phen, treatment with drought stress and o^{-}Phen (by spraying 1 mM) simultaneously

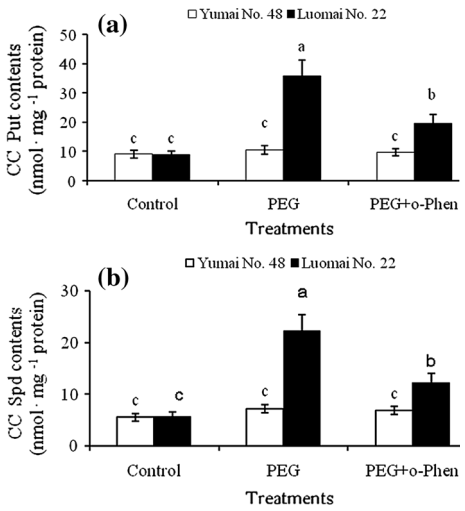

Fig. 5 Effects of PEG and o^{-}Phen on CC Put and CC Spd contents in PM vesicles from wheat embryos developing on MS solid medium, in vitro. On the 10th day after fertilization, young wheat embryos were taken out from developing seed and inoculated onto MS solid medium. After cultivation for 3 d, the embryos were treated with PEG (−0.5 MPa) and PEG (−0.5 MPa) + o^{-}Phen (0.5 mM) for 2 d, respectively, and control embryos were kept on MS solid medium without PEG or o^{-}Phen. Then, the treated and control embryos were sampled for assays of CC PAs in PM. Each value in the figure represents the mean of three experiments ± SE (standard error), *error bars* indicate SE (n = 9), and *different letters* (*a–c*) above the column are significantly different at $P < 0.05$ (Duncan's multiple range tests). PEG, treatment with PEG; PEG + o^{-}Phen, treatment with PEG and o^{-}Phen simultaneously

solid medium as experiment material. Following PEG treatment for 2 d, NCC Spd (Fig. 3b) and NCC Spm (Fig. 3c) in PM vesicles from embryos of Luomai No. 22 cv. increased more significantly than those of Yumai No. 48 cv. Exogenous Spd enhanced the osmotic-induced increase in NCC Spd (Fig. 3b) and NCC Spm (Fig. 3c) contents of Yumai No. 48 cv., and MGBG inhibited the osmotic-induced increase in NCC Spd (Fig. 3b) and NCC Spm (Fig. 3c) contents of Luomai No. 22 cv. (Fig. 3). The results, in vitro, were consistent with those, in vivo.

Effects of drought stress and o^{-}Phen on CC PAs contents in PM vesicles from developing wheat embryos

The contents of CC Put and CC Spd could be detected in PM vesicles purified from embryos of both cultivars under drought stress (Fig. 4), while the content of CC Spm was too low to be detected. Drought stress treatment markedly elevated the contents of CC Put (Fig. 4a) and CC Spd (Fig. 4b) of Luomai No. 22, but had a little effect on the levels of CC Put (Fig. 4a) and CC Spd of Yumai No. 48 (Fig. 4b). The treatment with o^{-}Phen inhibited the drought stress-induced increases in CC Put (Fig. 4a) and CC Spd (Fig. 4b) contents in PM from the embryos of both cultivars and the effect of o^{-}Phen on drought-treated Luomai No. 22 was greater than that observed on drought-treated Yumai No. 48 (Fig. 4).

Effects of PEG and o^{-}Phen on CC PA contents in PM vesicles from wheat embryos developing on MS solid medium, in vitro

To verify further the changes in CC PAs from PM vesicles, we used wheat embryos developing on MS solid medium as experiment material. Following PEG treatment for 2 d, CC Put (Fig. 5a) and CC Spd (Fig. 5b) in PM vesicles from the embryos of Luomai No. 22 cv. increased more significantly than those of Yumai No. 48 cv. O^{-}Phen inhibited markedly the osmotic-induced increase in CC Put and CC Spd contents of Luomai No. 22 cv. (Fig. 5). These results in vitro were consistent with those, in vivo, as noted above.

Effects of drought stress, exogenous Spd, MGBG and o^{-}Phen on PM H^{+}-ATPase activity of developing wheat embryos

PM-H^{+}-ATPase activity in embryos of Luomai No. 22 increased more significantly than that of Yumai No. 48

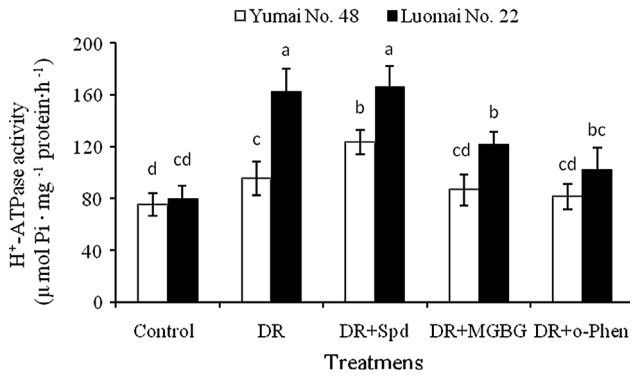

Fig. 6 Effects of drought stress, exogenous Spd, MGBG and o$^-$Phen on PM H$^+$-ATPase activity of developing wheat embryos. On the 10th day after fertilization, the wheat roots were treated under −1.0 MPa soil drought stress and the wheat roots with water–normal soil were as control group. The wheat flag leaves and ears were sprayed with Spd (1 mM), MGBG (1 mM) and o$^-$Phen (1 mM), respectively. The control group was sprayed with distilled water. After treatment for 3 d, the developing seed embryos were sampled and tested. Each value in the figure represents the mean of three experiments ±SE (standard error), *error bars* indicate SE (n = 9), and *different letters* (*a–d*) above the column are significantly different at $P < 0.05$ (Duncan's multiple range tests). DR, drought stress treatment; DR + Spd, treatment with drought stress and Spd (by spraying 1 mM) simultaneously; DR + MGBG, treatment with drought stress and MGBG (by spraying 1 mM) simultaneously; DR + o$^-$Phen, treatment with drought stress and o$^-$Phen (by spraying 1 mM) simultaneously

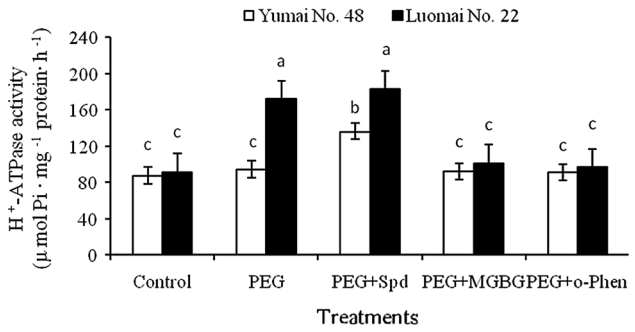

Fig. 7 Effects of PEG, exogenous Spd, MGBG and o$^-$Phen on PM H$^+$-ATPase activity from wheat embryos developing on MS solid medium, in vitro. On the 10th day after fertilization, young wheat embryos were taken out from developing seed and inoculated onto MS solid medium. After cultivation for 3 d, the embryos were treated with PEG (−0.5 MPa), PEG (−0.5 MPa) + Spd (0.5 mM), PEG (−0.5 MPa) + MGBG (0.5 mM), and PEG (−0.5 MPa) + o$^-$Phen (0.5 mM) for 2 d, respectively and control embryos were kept on MS solid medium without PEG, Spd, MGBG or o$^-$Phen. Then, the treated and control embryos were sampled for assays of H$^+$-ATPase activity in PM. Each value in the figure represents the mean of three experiments ± SE (standard error), *error bars* indicate SE (n = 9), and *different letters* (*a–c*) above the column are significantly different at $P < 0.05$ (Duncan's multiple range tests). PEG, treatment with PEG; PEG + Spd, treatment with PEG and Spd simultaneously; PEG + MGBG, treatment with PEG and MGBG simultaneously; PEG + o$^-$Phen, treatment with PEG and o$^-$Phen simultaneously

(Fig. 6) in response to drought stress. Exogenous Spd treatment enhanced the increase of PM-H$^+$-ATPase activity in embryos of Yumai No. 48 subjected to drought stress, and MGBG and o$^-$Phen treatments inhibited the drought stress-induced increases of PM-H$^+$-ATPase activity in Luomai No. 22 embryos to a markedly greater extent than in Yumai No. 48 (Fig. 6).

Effects of PEG, exogenous Spd, MGBG and o$^-$Phen on PM H$^+$-ATPase activity from wheat embryos developing on MS solid medium, in vitro

To verify further the change in PM-H$^+$-ATPase activity from embryos, we used wheat embryos developing on MS solid medium as experiment material. Following PEG treatment for 2 d, PM-H$^+$-ATPase activity in embryos of Luomai No. 22 increased more markedly than that of Yumai No. 48 (Fig. 7). Under PEG stress, the treatment with exogenous Spd increased PM-H$^+$-ATPase activity in the embryos of Yumai No. 48 more than in the embryos of Luomai No. 22, while MGBG and o$^-$Phen inhibited markly the osmotic-induced increases of PM H$^+$-ATPase activity in Luomai No. 22 (Fig. 7). These results, in vitro, were in accord with those, in vivo, as mentioned above.

Discussion

Relationship between drought stress and the contents of two forms of conjugated PAs in PM from developing wheat embryos under drought stress

It has been well-documented that PAs are associated with plant tolerance to various abiotic stress (Liu et al. 2004; Goyal and Asthir 2010; Qiao et al. 2012; Do et al. 2013; Grzesiak et al. 2013; Li et al. 2013). Furthermore, PAs are essential for normal seed development and active polyamine metabolism occurs in this period (Astarita et al. 2003; Uranoa et al. 2005; Cao et al. 2010). Because of their poly-cationic nature at physiological pH, PAs bind strongly to negative charges in cellular components such as nucleic acids, proteins, and phospholipids (Gupta et al. 2013) to form NCC PAs. In the present research, it was suggested that NCC Spd and NCC Spm might involve in the tolerance of wheat embryos to drought stress (Fig. 2). The outcome of additional experiments with exogenous Spd and MGBG treatments supported this suggestion (Fig. 2). The results of experiments, in vitro, also corroborated the suggestion (Fig. 3). Our finding is consistent with previously published findings mentioned below. With more positive charges, Spd and Spm bind noncovalently to protein and phospholipids more easily than Put (Dutra et al. 2013). Despite the fact that the precise role of Put in

environmental stress tolerance is still controversial, there are many reports emphasizing the protective role for Spd and Spm against drought stress in plants (Yamaguchi et al. 2007; Kubis 2008). Farooq et al. (2009) have argued that among the PAs, Spm is the most effective in improving drought tolerance. However, more in depth study is required to come to a comprehensive conclusion regarding regulation of PA metabolism in drought stress.

In plants, other than free and NCC PAs, PAs could covalently link to endoglutamines of proteins by action of TGase to be transformed into CC PAs, which stabilize the configuration and function of proteins (Del Duca et al. 1995). In this work, we observed that drought stress brought about more significant increases in CC Put and CC Spd levels in PM of drought-tolerant wheat embryos (Fig. 4), which is indicative of possible involvement of CC Put and CC Spd in the tolerance of wheat embryos to drought stress. The outcome of the additional experiment with o−Phen treatment supported this finding (Fig. 4) and the results of experiments, in vitro, also corroborated it (Fig. 5). The finding of Del Duca et al. (1995) is consistent with ours. To our knowledge, the exact mechanism by which CC PAs mediate plant growth and development under drought stress remains be ascertained. Therefore, in the future, with the availability of cutting-edge imaging and genomic techniques, the study of the arena would be interesting and significant.

Relationship between the activity of H^+-ATPase and the contents of two forms of conjugated PAs in PM from developing wheat embryos under drought stress

To further elucidate the function of the two conjugated PAs, the activity of H^+-ATPase in PM was detected. In the present research, drought treatment brought about a more significant increase of H+-ATPase activity in PM from drought-tolerant Luomai No. 22 embryos than that from drought-sensitive Yumai No. 48 (Fig. 6). The result suggested a possible involvement of PM H^+-ATPase in drought stress tolerance of wheat embryos. The suggestion is consistent with the result of previous work (Surowy and Boyer 1991; Michelet et al. 1994).

The regulatory mechanism of H^+-ATPase activity needs to be further studied. There have been extensive researches concerning the capacity of PAs to enhance the membrane-associated enzyme activities (Srivastava and Rajbabu 1983; Reggiani et al. 1992; Lester 2000; Janicka-Russak et al. 2010), and Zepeda-Jazo et al. (2011) reported that PAs improve Ca^{2+} and K^+ transport functions. The regulatory mechanism by which PAs regulate H^+-ATPase activity remains to be answered. In our research, we found that drought treatment brought about significant increases of not only the contents of NCC Spd, NCC Spm (Fig. 2), CC Put and CC Spd (Fig. 4), but also H^+-ATPase activity (Fig. 6) in PM of the drought-tolerant Luomai No. 22 embryos. These results are indicative of possible involvement of NCC Spd, NCC Spm, CC Put and CC Spd in the association with H^+-ATPase activity in PM. This notion was supported by the results of additional experiments with the treatments of exogenous Spd and inhibitors, MGBG and o$^-$Phen (Figs. 2, 4, 6). The results of the experiments, in vitro (Figs. 3, 5, 7), were consistent with the results noted above. Statistical analysis indicated that the sum of NCC Spd and NCC Spm was markedly positively correlated with the H^+-ATPase activities in PM ($r_{0.05} = 0.95$, $r_{0.01} = 0.83$, $n = 8$), and statistical analysis indicated that there was a markedly positive correlation between H^+-ATPase activity and the sum of contents of CC Put and CC Spd in PM ($r_{0.05} = 0.82$, $r_{0.01} = 0.81$, $n = 6$).

The reason why NCC Spd and NCC Spm, but not NCC Put could enhance the H^+-ATPase activity in PM is to be attributed to their cationic nature at physiological pH, as suggested by Sood and Nagar (2003). With more positive charges, NCC Spd and NCC Spm could modulate H^+-ATPase activity by noncovalently binding to protein more easily to affect its configuration and function than NCC Put. The *C*-terminal end of H^+-ATPase in PM plays a role as an auto-inhibitory regulatory domain (Palmgren et al. 1991; Johansson et al. 1993; Svennelid et al. 1999; Jelich et al. 2001) and it is hypothesized that factors which increase H^+-ATPase activity are likely to have the domain as their ultimate target. For example, H^+-ATPase in PM is activated due to the binding of 14-3-3 protein to its *C*-terminal amino acids (Svennelid et al. 1999; Jelich et al. 2001). Furthermore, it is suggested that PAs, especially Spd and Spm, could activate 14-3-3 protein by noncovalently binding acidic residues in the loop 8 of 14-3-3 protein to neutralize the negative charge (Athwal and Huber 2002). Our finding is consistent with the researches of Garufi et al. (2007) and Shen and Huber (2006), which revealed that among the different PAs, Spm brings about more stimulation of the H^+-ATPase activity and this effect is due to an increase in 14-3-3 levels associated with the enzyme. This implies that Spd and Spm with more positive charges bind noncovalently to 14-3-3 protein more easily. However, the report of Dutra et al. (2013) indicated that PAs reduces the activities of proton pumps, such as H+-ATPase in embryogenic suspension cultures of *Araucaria angustifolia*. The diversity might be attributed to different plant species, organ, tissue, and the development stage. Although studies on the mode of action of PAs on the proton pumps remain to be in-depth in plants, we can suggest that PAs could modulate the activity of proton pumps due to its poly-cationic nature, involving a phosphorylation cascade and 14-3-3 dependent modulation, which is also complicated. For example, Camoni et al.

(2012) reported that binding of phosphatidic acid to 14-3-3 proteins hampers their ability to activate the plant PM H^+-ATPase. Besides the reason discussed above, Spd and Spm might affect the PM's physical state by noncovalently conjugating to membrane phospholipids with negative charges. The change in the PM physical state is associated with H^+-ATPase activity in PM (Zhang et al. 2002).

As to CC PAs, it is reported (Del Duca et al. 1995) that PAs could have an important function in chloroplasts by being covalently conjugated to endo-glutamyl residues of the chlorophyll *a*/*b* antenna complex, CP26, CP24, CP26 and the large subunit of Rubisco. The conversion of free PAs to CC PAs, forming protein-Glu-PAs and protein–Glu-PAs-protein, could stabilize the configuration and function of PM proteins (including H^+-ATPase, of course) by preventing these proteins from denaturing under drought stress and thus promote the activity of these enzymes. Obviously, the effect of conjugated PAs on H^+-ATPase activity in PM is complex, interesting, and deserves further investigation. Further studies underling the proton pumps modulation by PAs would shade new light on the mechanism of action in plants under various stresses.

Summarily, to our knowledge, the present research is the first to show that the elevated NCC Spd, NCC Spm, together with CC Put and CC Spd induced by drought stress could promote H^+-ATPase activity in PM, and via doing so, might enhance the tolerance of developing wheat embryos to drought stress. It is very well-known that PAs are present in all the plant cell compartments, which is a proof of its indispensable nature and role in diverse cellular processes (Kumer et al. 1997; Kaur-Sawhney et al. 2003; Legocka and Sobieszczuk-Nowicka, 2012). Further research is needed to ascertain the subcellular distribution of all forms of PAs, configuration and function of biomacromolecule in cell treated with drought stress, exogenous PAs, and the application of PA biosynthesis inhibitor.

Acknowledgments This research was supported by the National Natural Science Foundation of China (Grant No. 31271627), the Special Fund of Industrial (Agriculture) Research for Public Welfare of China (Grant No. 201203077) and the Education Department Natural Science Foundation of Henan Province (Grant No. 2011A180033).

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