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Improved oxidative tolerance in suspension-cultured cells of C_4-*pepc*transgenic rice by H_2O_2 and Ca^{2+} under PEG-6000

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Research Article

Abstract To understand the molecular responses of PC (Overexpressing the maize C_4-*pepc* gene, which encodes phosphoenolpyruvate carboxylase (PEPC)), to drought stress at cell level, we analyzed changes in the levels of signaling molecules (hydrogen peroxide (H_2O_2), calcium ion (Ca^{2+}), and nitric oxide (NO)) in suspension-cultured PC and wild-type (WT) rice (*Oryza sativa* L.) cell under drought stress induced by 20% polyethylene glycol 6000 (PEG-6000). Results demonstrated that PC improved drought tolerance by enhancing antioxidant defense, retaining higher relative water content, survival percentages, and dry weight of cells. In addition, PEPC activity in PC under PEG treatment was strengthened by addition of H_2O_2 inhibitor, dimethylthiourea (DMTU) and NO synthesis inhibitor, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), respectively, while that in PC was weakened by addition of free calcium chelator, ethylene glycol-bis(b-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) + calcium channel outflow inhibitor, ruthenium red (RR) + plasma membrane channel blocker $La(NO_3)_3$, but EGTA + RR did not. Results also showed that NO and Ca^{2+} was lying downstream of H_2O_2 in drought-induced signaling. Calcium ion was also involved in the expression of C_4-*pepc* in PC. These results suggested that PC could improve oxidative tolerance in suspension-cultured cells and the acquisition of this tolerance required downregulation of H_2O_2 and the entry of extracellular Ca^{2+} into cells across the plasma membrane for regulation of PEPC activity and C_4-*pepc* expression.

Keywords: Calcium; drought; hydrogen peroxide; phosphoenolpyruvate carboxylase; rice (*Oryza sativa* L.)
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INTRODUCTION

Under conditions of high light intensity, low CO_2 concentrations, and drought, C_4 plants, which have a unique CO_2-concentrating mechanism, have higher photosynthetic capacity and higher nitrogen and water use efficiencies than those of C_3 plants (Zhu et al. 2010). Carbon dioxide fixation in a C_3 plant such as rice (*Oryza sativa* L.) is catalyzed by the enzyme RuBisCO (ribulose-1,5-bisphosphate carboxylase-oxygenase). RuBisCO also catalyzes the reaction with O_2 resulting in photorespiration. The C_4 photosynthetic pathway has evolved to virtually eliminate oxygenase activity by RuBisCO. This is achieved by bicarbonate reacting with phosphoenolpyruvate, PEP, a carboxylation reaction insensitive to O_2. The C_4 acids formed in the mesophyll cells diffuse into bundle sheath cells where they are decarboxylated. A diffusive resistance restricts CO_2 leakage out of the bundle sheath such that the CO_2 partial pressure in the bundle sheath cells is greatly increased, resulting in the suppression of photorespiration. These features are described for a typical type of C_4 plant such as maize (*Zea mays* L.) (Hatch 1987). Phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) is one of the key enzymes in the C_4 photosynthetic pathway. Increases in PEPC activity can be induced by stresses such as drought, salt, and cold, and this enzyme plays a role in the resilience of C_4 plants in diverse environments (Sánchez et al. 2006; Doubnerová and Ryšlaváa 2011; Doubnerová et al. 2014). Through transgenic technology, the C_4-*pepc* gene from maize has been successfully introduced into rice, and it was expressed at high levels (Ku et al. 1999). Research on transgenic rice lines expressing high levels of C_4-*pepc* (hereafter, PC lines) showed that, compared with wild-type (WT) rice, the PC lines had a higher net photosynthetic rate (Pn) and carboxylation efficiency, and a lower CO_2 compensation point (Li et al. 2005). The PC lines also showed greater tolerance than that of WT to photo-oxidation, as indicated by a slower inhibition of F_V/F_M and Pn, and lower levels of reactive oxygen species (ROS) generation (Jiao et al. 2005). Furthermore, compared with WT, the PC lines showed elevated sucrose synthetase (EC 2.4.1.13) activity in the leaves during the flowering stage, and increased activities of some other nitrogen metabolism enzymes, including nitrate reductase (EC 1.6.6.1), 2-oxaloacetic acid aminotransferase (EC 2.6.1.1), alanine aminotransferase (EC 2.6.1.2), glutamine synthetase (EC 6.3.1.2), and asparagine synthetase (EC 6.3.1.1) (Li and Wang 2013). The PC plants were able to retain

a relatively high Pn under drought stress (Ding et al. 2012). However, the molecular mechanisms of drought tolerance in PC remain unclear at the cellular level.

Under drought stress, various ROS are generated in plants, and hydrogen peroxide (H_2O_2) is the main component (Mittler et al. 2011). In many previous studies, H_2O_2 has been shown to participate in the initiation of many biotic and abiotic stress signals (Gill and Tuteja 2010; Kim et al. 2010). Nitric oxide (NO) is a free radical generated in plant cells that belongs to a class of reactive nitrogen species (Corpasa et al. 2007). Unfavorable environmental conditions cause an imbalance in reactive nitrogen species in plants. This imbalance directly or indirectly leads to cellular or molecular damage (Corpasa et al. 2011). Hydrogen peroxide and NO are forms of reactive oxygen and reactive nitrogen species, respectively, having wide-ranging effects in many biological systems (Durner et al. 1999; Finkel and Holbrook 2000). Although the effects of both H_2O_2 and NO on plant physiology and development have been the subject of investigation for several years, it is only relatively recently that their roles as signaling molecules have been characterized more fully. The physiological effects of NO and H_2O_2 are similar and synergistic, which are both involved in regulating stomatal movements (Desikan et al. 2004). Cantrel et al. (2011) reported that NO participated in cold-responsive phosphosphingolipid formation and gene expression in *Arabidopsis thaliana*. Endogenous free calcium ions (Ca^{2+}) are known to play an important physiological role in plant growth and development. They function as a second messenger to regulate many physiological processes such as drought signal transmission via abscisic acid (ABA), regulation of the degree of opening and closing of stomata, and induction of stress-related gene expression (Dodd et al. 2010). As essential signaling molecules involved in regulating plant growth and development, H_2O_2, NO, and Ca^{2+} form a complex signaling network. During adventitious root formation in the mung bean, H_2O_2, and NO signals exhibited two parallel pathways, and the Ca^{2+} signal was downstream of both pathways (Li and Xue 2010). However, the relationships among these molecules in different physiological processes remain unclear.

In the complex signaling networks involved in regulating plant growth and development, H_2O_2, NO, and Ca^{2+} are located in the upstream part of signaling pathways, and they participate in regulating the expression and activity of different genes and enzymes. In our previous study, we detected significant differences in the levels of these signaling molecules between PC lines and WT (Li et al. 2011). Ren et al. (2014) also reported that the regulation of endogenous H_2O_2 metabolism via phosphatidic acid (PA) may play a role in enhancing photosynthetic capacity. Wang et al. (2012b) reported that PEPC activity was modulated by PA signals in suspension-cultured cells of *C_4-pepc* transgenic rice. The results also showed that changes in the level of PA in PC resulted in significant increases in the intracellular Ca^{2+} concentration, and there was a direct correlation between NO and H_2O_2 concentrations and PEPC activity under normal conditions. Chen et al. (2014) also reported that PEPC activity and *C_4-pepc* gene expression increased in response to exogenous NO donors, and that PC lines showed enhanced photosynthetic capacity via Ca^{2+} and PA under these conditions. Together, these results demonstrated there may be specific regulation of H_2O_2, NO, PA, and Ca^{2+} in PC lines. However, how PC can induce oxidative tolerance and the acquisition of oxidative tolerance is involved in these second messengers such as H_2O_2, NO, and Ca^{2+}, cross-talk among these messengers under stress conditions is poorly known. Polyethylene glycol compounds have been used to simulate water stress effect in plants (Murillo-Amadaor et al. 2002). In this study, we investigated the effects of drought which is created by application of polyethylene glycol 6000 (PEG-6000). We used suspension cells of PC and WT (*O. sativa* cv. Kitaake) as the experimental materials to explore the mechanisms underlying the response to drought at the cellular level. We assumed that under drought stress, there would be an initial increase in the endogenous H_2O_2 content in PC at an early stage after perceiving the drought stimulus, with a concomitant downregulation of Ca^{2+}. These changes would promote an increase in PEPC activity and the antioxidant system via upregulation of transcription factors that affect the expressions of genes involved in oxidative tolerance under drought conditions.

The study provides important information about the stress tolerance mechanisms conferred by a C_4 photosynthesis gene at the cellular level, and will be useful to optimize production of "C_4 rice" in the future.

RESULTS

Growth curve of suspension cells and survival rate of suspension cells under 20% PEG-6000 treatment

Figure 1 shows the growth of suspension cells of WT and PC over 14 d, measured as fresh weight (FW) and dry weight (DW). The peak in FW was on day 6 for both WT and PC (Figure 1A). The highest DW values were recorded on day 6 and day 8 (Figure 1B). After PEG-6000 treatments, the FW both in WT and PC had no different changes than in those in CK, while the DW both in WT and PC after PEG-6000 treatment was decreased obviously. Furthermore, the DWs in PC were decreased less than those in WT.

Suspension cells cultured for 5 d in logarithmic phase were used for the following experimental materials. Five day old cultured cells were exposed to PEG-6000 for 8 h in a rotary shaker. To evaluate whether the suspension cells were seriously damaged by this treatment, the integrity of the cells was assessed by observation under a microscope after staining with 0.04% Trypan blue. After 8 h of the 20% PEG-6000 treatment, the membranes of cells were still intact both in WT and PC (Figure 2). But PEG treatment did have significant ($P < 0.05$) effects on survival percentage of suspension cells both in WT and PC, illustrating that PEG had toxic effect on cells of rice (Figure 3). Obviously, the survival percentage of suspension cells in PC was higher than that in WT under PEG treatment.

In addition to survival percentage, further experiments exhibited that PEG treatments alleviated reduced accumulation of indicator of membrane lipid peroxidation, malondialdehyde (MDA) (Figure 4A), and decrease in relative water content (RWC) of PC (Figure 4B) compared with those of WT. These results indicated that the PC suspension cells were less damaged than WT cells by the 20% PEG-6000 treatment.

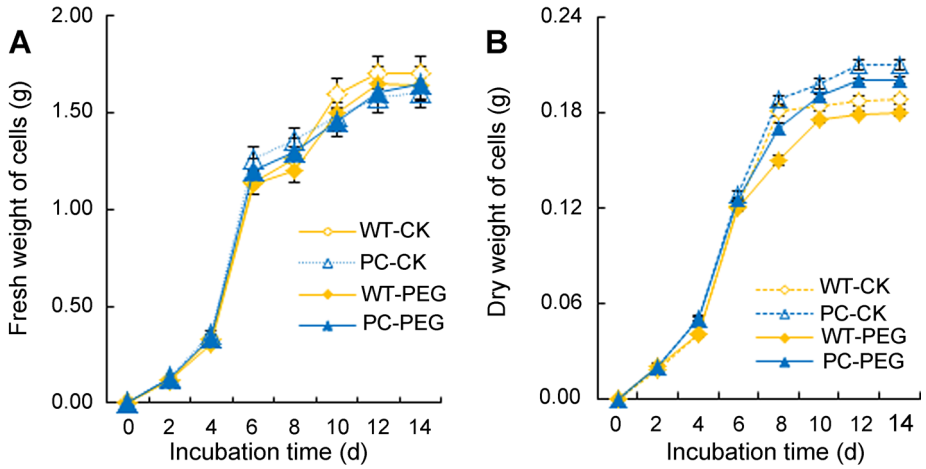

Figure 1. Growth curve of cell suspensions of C_4-*pepc*-transformed (PC) and wild-type (WT) rice for tested materials under normal and drought conditions

(A) Fresh weight. **(B)** Dry weight. The suspension cells were subjected to 20% polyethylene glycol 6000 (PEG-6000) treatments at 28 °C for 14 d, and then weighed the fresh weight and dry weight of suspension cells per 2 d. Error bar represent *SE* and each date represents the mean ± *SE* of three experiments.

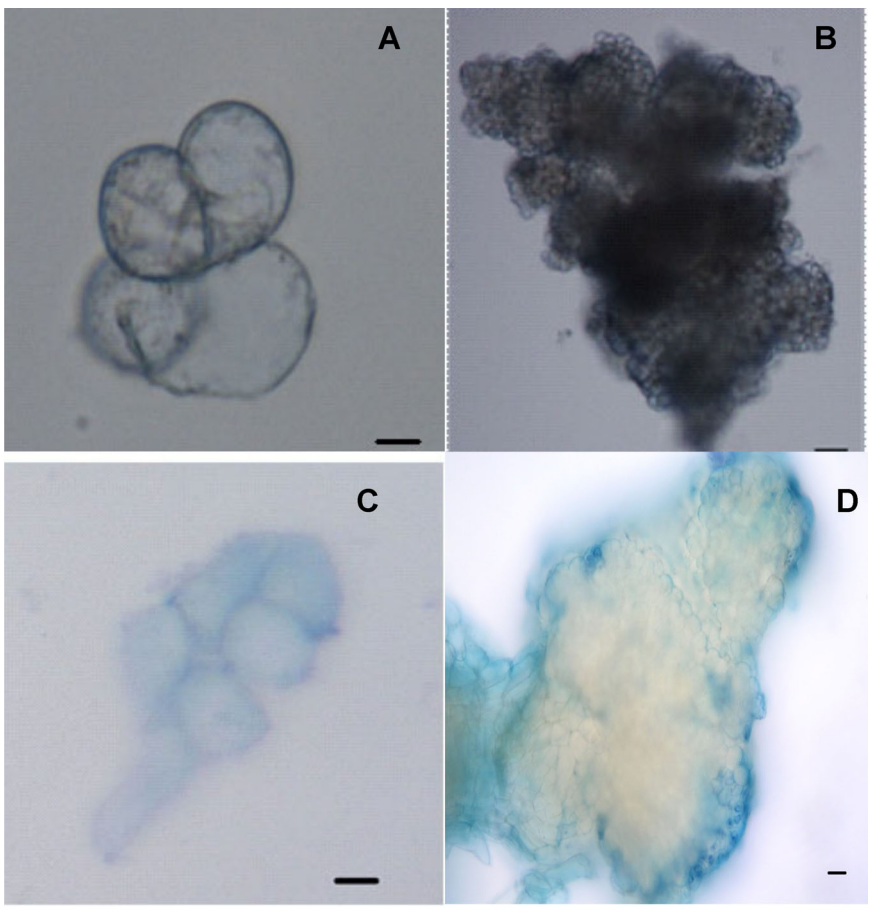

Figure 2. The integrity of cell suspensions of C_4-*pepc*-transformed (PC: A, B) and wild-type (WT: C, D) rice under 20% polyethylene glycol 6000 (PEG-6000) treatment for 12 h

(A, C) Single cell suspension, bar = 10 μm. **(B, D)** Cell suspensions mass, bar = 50 μm.

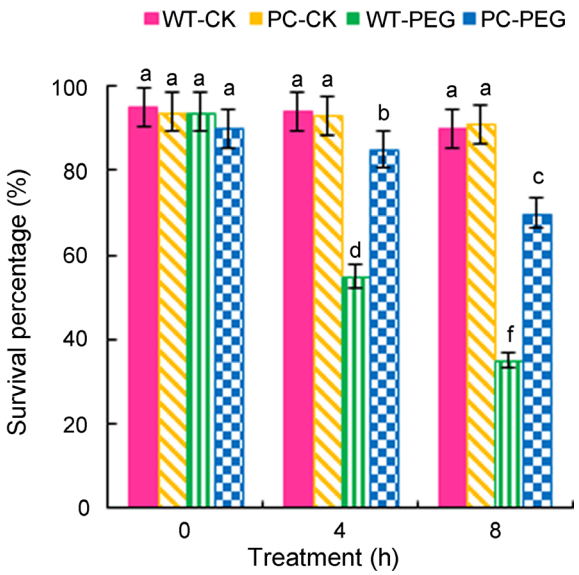

Figure 3. Changes of survival percentage of rice suspension-cultured cells of C_4-*pepc*-transformed (PC) and wild-type (WT) rice under polyethylene glycol 6000 (PEG-6000) treatment

Five day old suspension cells were subjected to 20% polyethylene glycol 6000 (PEG-6000) treatments at 28 °C for 8 h, and then survival percentage of suspension cells was determined. Error bar represents *SE* and each date represents the mean ± *SE* of three experiments. Bars with different letters were significantly different at the $P < 0.05$ (lowercase) or $P < 0.01$ (uppercase) level according to Duncan's multiple range test.

PC decreased ROS accumulation and improved SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), and APX (EC 1.11.1.11) activity under PEG-6000 treatment

PC had lower MDA levels than WT under drought stress (Figure 4A), implying that they may be subjected to less serious oxidative injury than the WT. Therefore, it was of interest to detect ROS accumulation in the PC lines and WT. Enzymatic antioxidants play important roles in ROS scavenging and influence cellular ROS balance (Hu et al. 2013). Therefore, the activities of three significant antioxidant enzymes were measured in the suspension cells. In CK conditions, activities of superoxide dismutase (SOD) in PC transgenic lines were higher than in WT (Figure 5A). After 4 and 8 h of drought stress, PC displayed significantly higher SOD activities than WT (Figure 5A). There is no significant difference in catalase (CAT) activity between PC lines and WT with or without drought stress (Figure 5B). In CK conditions, activities of ascorbate peroxidase (APX) in PC transgenic lines were lower than in WT (Figure 5C). While after 4 and 8 h of drought stress, PC displayed significantly higher APX activities than WT (Figure 5C). These results suggested that overexpression of C_4-*pepc* may reduce ROS accumulation by enhancing SOD and APX activities under drought stress.

Changes in H_2O_2, NO, and Ca^{2+} contents in suspension cells under 20% PEG-6000 treatment

In plant cells, some messengers such as H_2O_2, NO, and Ca^{2+} are involved in the acquisition of various stress tolerance as identified using pharmacological and other biological methods (Desikan et al. 2004). To investigate the effects of H_2O_2, NO, and Ca^{2+} on PEG-induced oxidative tolerance in PC, PEG treatments in combination with dimethylthiourea (DMTU), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), ethylene glycol tetraacetic acid (EGTA),

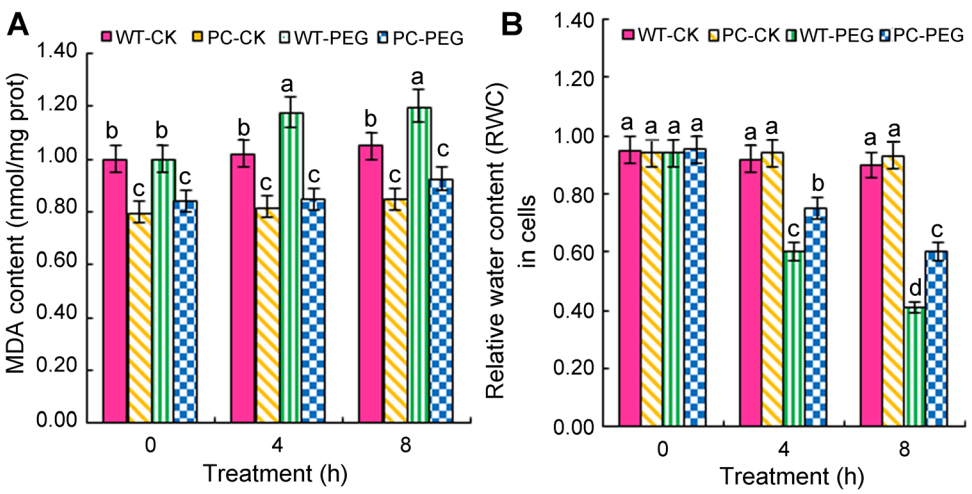

Figure 4. Analysis of malondialdehyde (MDA) (A) and relative water content (RWC) (B) of C_4-*pepc*-transformed (PC) and wild-type (WT) rice under normal and drought conditions

Five day old suspension cells were subjected to 20% polyethylene glycol 6000 (PEG-6000) treatments at 28 °C for 8 h. After treating for 0, 4, and 8 h, suspension cells were collected to measure MDA and RWC. Error bar represent *SE* and each date represents the mean ± *SE* of three experiments. Bars with different letters were significantly different at the $P < 0.05$ (lowercase) or $P < 0.01$ (uppercase) level according to Duncan's multiple range test.

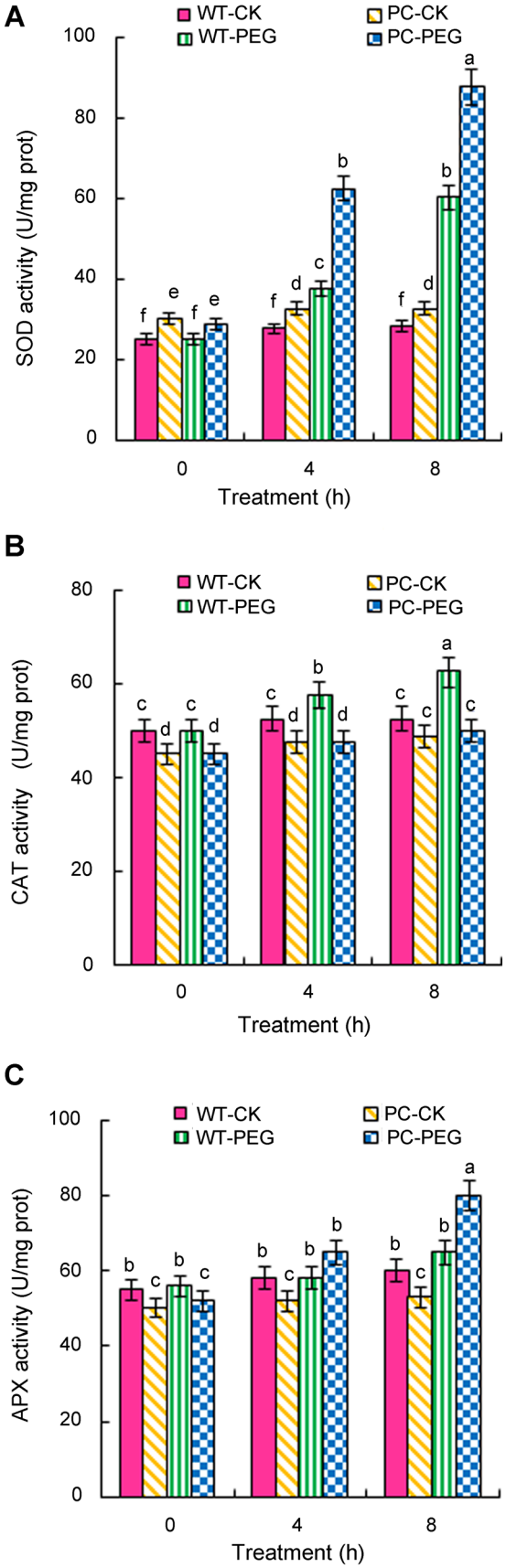

Figure 5. Continued.

ruthenium red (RR), $La(NO_3)_3$ were investigated, respectively, and the results indicated that, in normal culture conditions at 28 °C, chemical alone or combination treatment had no effect on the content of H_2O_2 of rice suspension cells (Figure 6C). At PEG-6000 treatment, we analyzed the levels of signaling molecules in PC and WT suspension cells. The H_2O_2 content in PC and WT suspension cells were increased in response to 20% PEG stress; the H_2O_2 content in WT was significantly increased than in those in PC suspension cells after 4 and 8 h of the PEG-6000 treatment. In contrast, PEG-induced H_2O_2 increases in PC and WT were significantly weakened by application of the exogenous H_2O_2 inhibitor, DMTU, compared with control (Figure 6C, $P < 0.05$). There are no significant effects in the increase of H_2O_2 content both in PC lines and WT by application of the exogenous NO synthesis inhibitor, cPTIO, a free calcium chelator, EGTA, a calcium channel outflow inhibitor, RR, and a plasma membrane channel blocker, $La(NO_3)_3$ (Figure 6D–F). These results suggested that the production of H_2O_2 was an upstream step before NO or Ca^{2+} accumulations in antioxidant defense in plant cells by drought-stress signaling. One concern in our results is that we still observed the lower levels of H_2O_2 contents in PC by application of the above-mentioned chemical reagents, compared with those in WT (Figure 6).

In normal culture conditions at 28 °C, there was no significant difference in NO contents between PC lines and WT (Figure 7A). The NO contents were increased in both PC and WT cells under PEG-6000 stress, compared with control (Figure 7B, $P < 0.05$). There was a marked increase in NO content in PC and WT cells by application of DMTU after 4 and 8 h of the PEG-6000 treatment, compared with their controls (Figure 7C, $P < 0.05$). In contrast, PEG-induced NO increases in PC and WT cells after 4 and 8 h of the PEG-6000 treatment were significantly weakened by application of the exogenous NO synthesis inhibitor, cPTIO, compared with control (Figure 6D, $P < 0.05$). It seems that there may be an NO pathway independent of H_2O_2 in response to drought stress. The effects of Ca^{2+} on NO content (Figure 7E, F) indicated that NO content was more related to the influx of extracellular calcium under PEG-6000 stress (Figure 7F, $P < 0.05$) observed in cells under PEG-6000 stress, especially for PC. These results suggested that the production of NO was a downstream step before Ca^{2+} accumulations in antioxidant defense in plant cells by drought-stress signaling.

As shown in Figure 8, we did not observe the difference in Ca^{2+} content in WT and PC cells under control conditions (Figure 8A); under PEG treatment, the Ca^{2+} content was

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Figure 5. Analysis of superoxide dismutase (SOD) (A) and catalase (CAT) (B) and ascorbate peroxidase (APX) (C) activities in suspension cells of the wild-type (WT) and PC under normal and drought conditions

Five day old suspension cells were subjected to 20% polyethylene glycol 6000 (PEG-6000) treatments at 28 °C for 8 h. After treating for 0, 4, and 8 h, suspension cells were collected to detect SOD, CAT, and APX activities. Error bar represents *SE* and each date represents the mean ± *SE* of three experiments. Bars with different letters were significantly different at the $P < 0.05$ (lowercase) or $P < 0.01$ (uppercase) level according to Duncan's multiple range test.

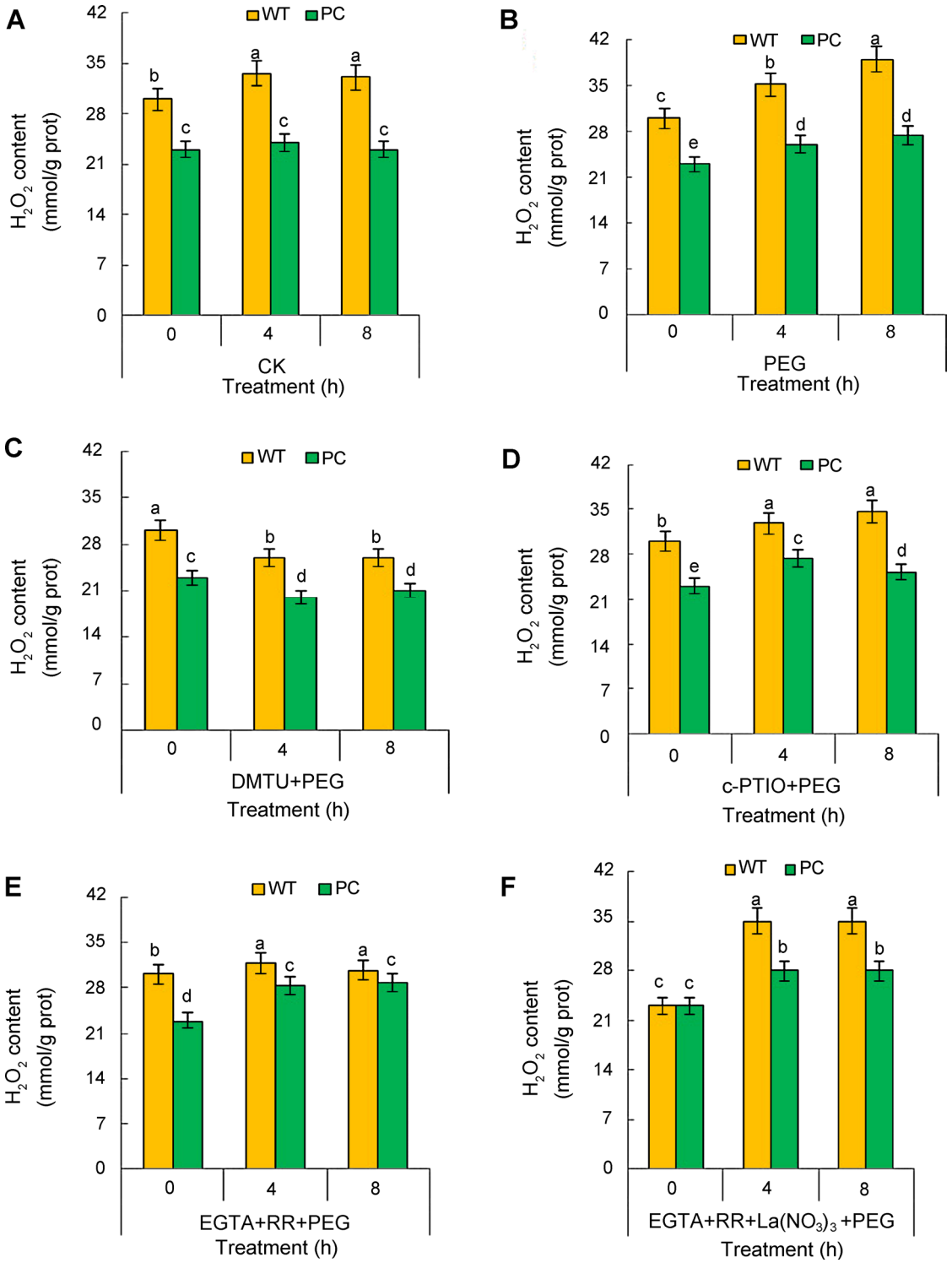


Figure 6. Effects of water (CK, not treated), polyethylene glycol (PEG), dimethylthiourea (DMTU), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), EGTA + RR, and EGTA + RR + $La(NO_3)_3$ on H_2O_2 content in suspension-cultured cells of C_4-*pepc*-transformed (PC) and wild-type (WT) rice

(A) CK, not treated. **(B)** PEG. **(C)** DMTU + PEG. **(D)** cPTIO + PEG. **(E)** EGTA + RR + PEG. **(F)** EGTA + RR + $La(NO_3)_3$ + PEG. Five-day-old suspension cells were subjected to 20% polyethylene glycol 6000 (PEG-6000) treatments at 28 °C for 8 h. After treating for 0, 4, and 8 h, suspension cells were collected to detect H_2O_2 content. Error bar represent *SE* and each date represents the mean ± *SE* of three experiments. Bars with different letters were significantly different at the $P < 0.05$ (lowercase) or $P < 0.01$ (uppercase) level according to Duncan's multiple range test.

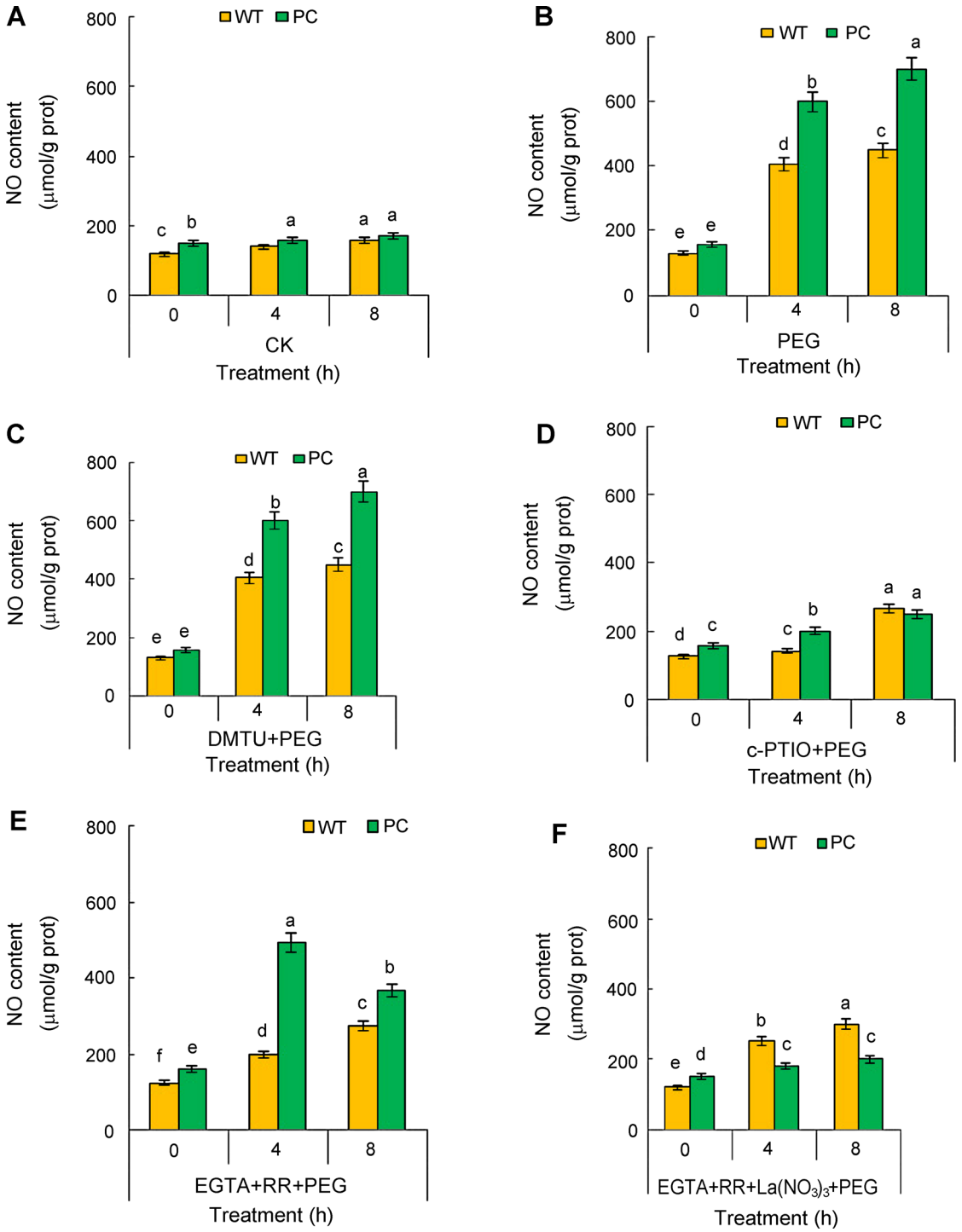


Figure 7. Effects of water (CK, not treated), polyethylene glycol (PEG), dimethylthiourea (DMTU), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), ethylene glycol-bis(b-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) + ruthenium red (RR), and EGTA + RR + $La(NO_3)_3$ on NO content in suspension-cultured cells of C_4-*pepc*-transformed (PC) and wild-type (WT) rice

(A) CK, not treated. **(B)** PEG. **(C)** DMTU + PEG. **(D)** cPTIO + PEG. **(E)** EGTA + RR + PEG. **(F)** EGTA + RR + $La(NO_3)_3$ + PEG. Five-day-old suspension cells were subjected to 20% polyethylene glycol 6000 (PEG-6000) treatments at 28 °C for 8 h. After treating for 0, 4, and 8 h, suspension cells were collected to detect NO content. Error bar represent *SE* and each date represents the mean ± *SE* of three experiments. Bars with different letters were significantly different at the $P < 0.05$ (lowercase) or $P < 0.01$ (uppercase) level according to Duncan's multiple range test.

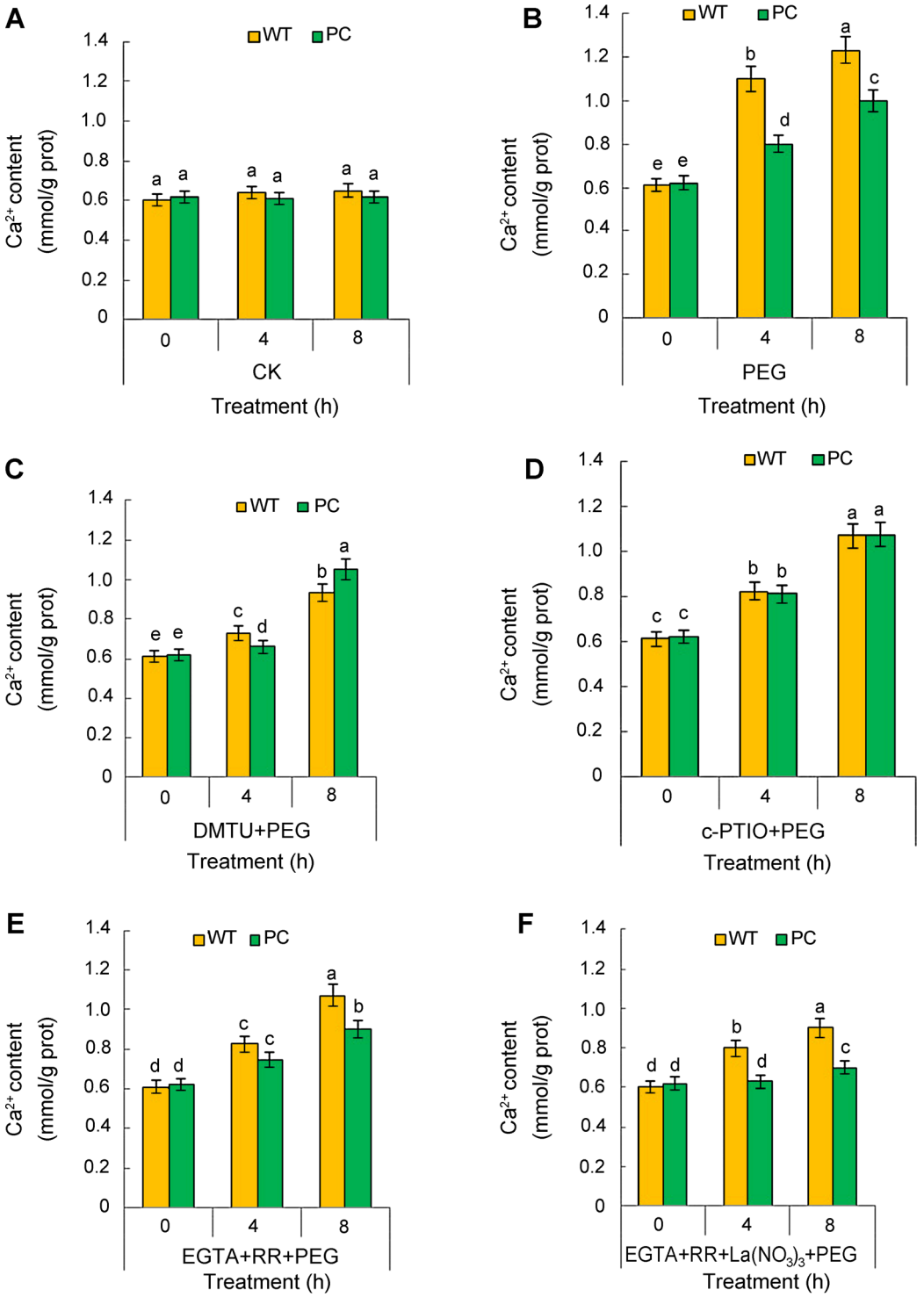


Figure 8. Effects of water (CK, not treated), PEG, dimethylthiourea (DMTU), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), ethylene glycol-bis(b-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) + ruthenium red (RR), and EGTA + RR + $La(NO_3)_3$ on Ca^{2+} content in suspension-cultured cells of C_4-*pepc*-transformed (PC) and wild-type (WT) rice

(A) CK, not treated. **(B)** PEG. (C) DMTU + PEG. **(D)** cPTIO + PEG. **(E)** EGTA + RR + PEG. (F) EGTA + RR + $La(NO_3)_3$ + PEG. Five day old suspension cells were subjected to 20% polyethylene glycol 6000 (PEG-6000) treatments at 28 °C for 8 h. After treating for 0, 4, and 8 h, suspension cells were collected to detect phosphoenolpyruvate carboxylase (PEPC) activity. Error bar represent *SE* and each date represents the mean ± *SE* of three experiments. Bars with different letters were significantly different at the $P < 0.05$ (lowercase) or $P < 0.01$ (uppercase) level according to Duncan's multiple range test.

increased both in WT and PC (Figure 8B). But the level of Ca^{2+} content in PC was much lower than that in WT. The Ca^{2+} content of cells in WT and PC were not affected by application of DMTU (Figure 8C) or cPTIO (Figure 8D). The decrease of Ca^{2+} level in PC by application of EGTA, RR, and $La(NO_3)_3$ was more than that in WT cells (Figure 8E, F).

H_2O_2 and Ca^{2+} signaling is involved in the PEPC activity in PC by PEG-6000 treatments

As shown in Figure 9A, the PEPC activity of cells in PC was much higher than that in both WT under control. After PEG-6000 treatment, the PEPC activities of PC and WT were both significantly increased after 4 and 8 h of the PEG-6000 treatment. Furthermore, the increase in PEPC activity of PC was much higher than that of WT (Figure 9B). These results suggested that high PEPC activity plays a role in antioxidant defense in cells of PC. By addition of DMTU, PEPC activity in PC after 8 h of the PEG-6000 treatment was markedly induced, suggesting the downregulation of H_2O_2 on PEPC activity (Figure 9C). Addition of cPTIO to the 20% PEG-6000 treatment resulted in a significant increase in PEPC in PC suspension cells. But these changes did not appear in WT in the same treatments (Figure 9D). These results indicated there may be a bypass on regulation of PEPC being independent of NO in PC (Figure 9D). Addition of EGTA and RR eliminated the induced increase of PEPC activity in PC, but that of WT after 8 h of the PEG-6000 treatment was significantly increased in WT (Figure 9E, $P < 0.05$). Upon further application of $La(NO_3)_3$ in combination with EGTA and RR under PEG treatment, the PEPC activity in PC was even decreased after 4 and 8 h of the PEG-6000 treatment. It can be seen that the inhibition of calcium influx on the plasma membrane significantly reduced the PEPC activity of PC under PEG treatment, suggesting the upregulation on PEPC activity by the calcium influx on the plasma membrane (Figure 9F).

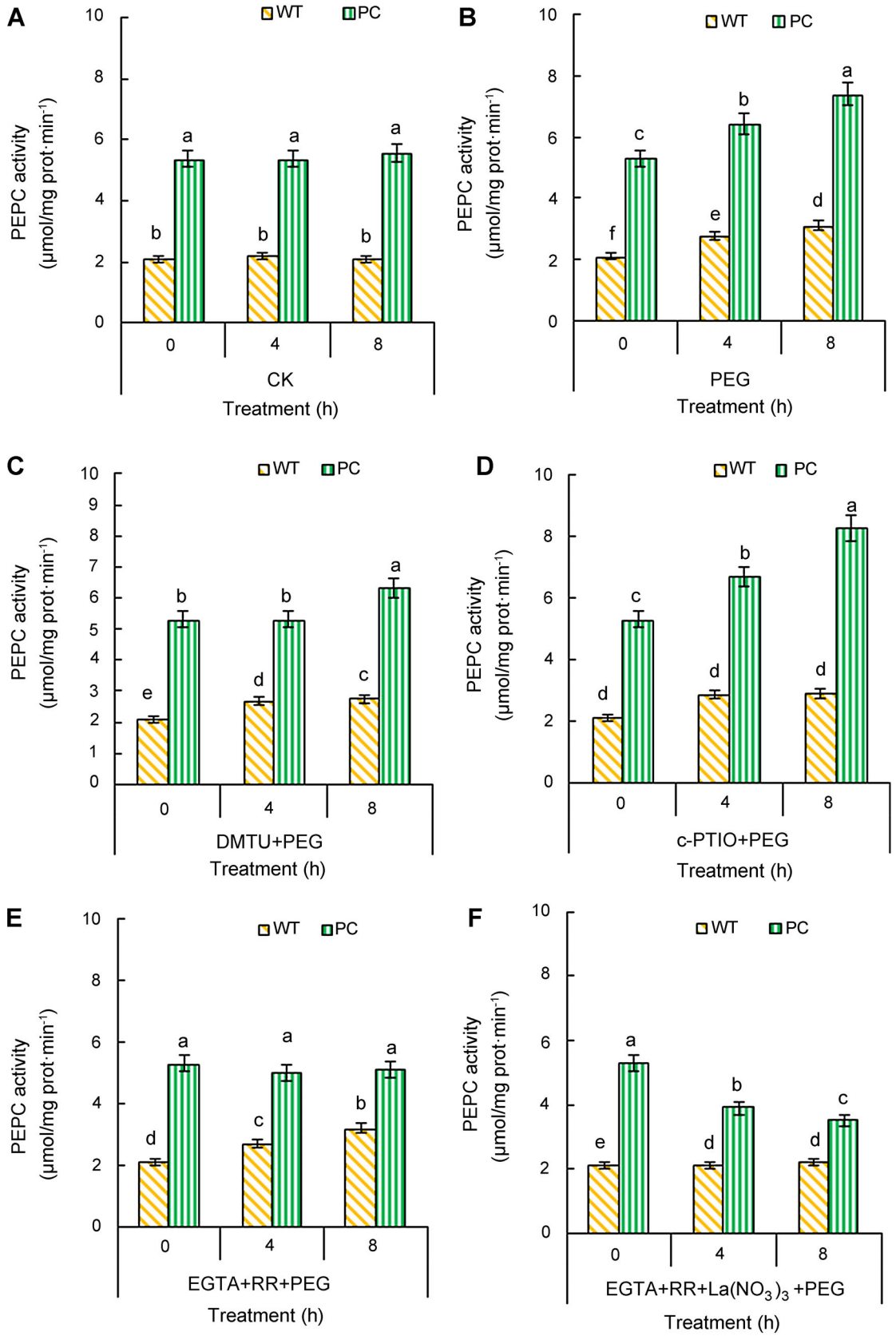
Transcript levels of some genes in suspension cells of PC after addition of various reagents to the 20% PEG-6000 treatment

As shown in Figure 10, PC contained the maize *C_4-pepc* gene, while WT did not. The quantitative real-time polymerase chain reaction (qRT-PCR) analysis revealed quantitative changes in *C_4-pepc*, *cu/zn-sod*, and *NAC6* transcript levels in response to the various treatments (Figure 11). Polyethylene glycol treatment increased *C_4-pepc*, *cu/zn-sod*, and *NAC6* transcript levels compared with that in the control (PC with no treatment). In the combined DMTU and PEG treatment, *C_4-pepc*, *cu/zn-sod*, and *NAC6* transcript levels were partly decreased compared with that in PEG treatment alone, but still higher than that of the control, suggesting that H_2O_2 is partly involved in the gene expressions in PC under PEG-6000 treatments. There were no significant changes in *C_4-pepc*, *cu/zn-sod*, and *NAC6* gene expression in PC after addition of the cPTIO under PEG treatment. But the combined PEG treatments with $La(NO_3)_3$, EGTA, and RR resulted in decreased *C_4-pepc* transcript levels in PC, compared with that in the control (PC with no treatment). But EGTA and RR under PEG treatment did not affect the expression of *cu/zn-sod* and *NAC6*. But the combined PEG treatments together with $La(NO_3)_3$, EGTA, and RR decreased the expression of the *cu/zn-sod* gene, compared with that of the PEG treatment. Interestingly, the transcription factor gene, *NAC6*, under the above-mentioned treatments still maintained higher transcript levels than that in the control (PC with no treatment), suggesting that the transcription factor gene *NAC6* may play a role before the regulation of the antioxidant enzymes and *C_4-pepc* gene in antioxidant defense in plant cells by drought stress. These results indicated that the increase of the transcription factors and antioxidant enzymes in PC also occurred, involved by H_2O_2 and Ca^{2+}, in the response to drought stress.

DISCUSSION

Compared with C_3 plants, C_4 plants have a higher photosynthetic capacity, and higher nitrogen and water use efficiencies under unfavorable conditions such as drought stress (Zhu et al. 2010). These advantages, in terms of stress tolerance, are closely related to PEPC activity (Caemmerer et al. 2012). Introducing the maize *C_4-pepc* gene into rice through transgenic technology enhanced the antioxidant capacity of the PC transgenic lines (Jiao et al. 2005; Ding et al. 2012; Ren et al. 2014). Lipid peroxidation is a common indicator of free radical reactions in tissues, and the amount of lipid peroxidation products indicates the extent of stress-induced injury (Sharma and Dubey 2005). The present study documents the induction of an oxidative burst in suspension-cultured rice cells transferred to PEG medium. We observed that the MDA content increased gradually from 0–4 to 8 h under PEG-6000 treatment in WT cells, but that in PC was less than in WT after 4 and 8 h in PC cells (Figure 4A). This result indicated that PC cells were less injured than WT cells by 4 h of the PEG-6000 treatment. The ROS level can also serve as an indicator of tolerance to oxidative stress, or it can reflect signaling of stress conditions in plants (Foyer and Noctor 2005). As shown in Figure 6A, H_2O_2 accumulation was markedly increased in both WT and PC cells after 4 and 8 h of PEG-6000 stress. However, the H_2O_2 accumulation in PC still kept a low level after 4 and 8 h of the PEG-6000 treatment, which was consistent with the increased SOD activity (Figure 5A) and APX (Figure 5C). This result further illustrated that the antioxidant capacity was greater in cells of PC than in WT under PEG stress, exhibiting a higher DW (Figure 1B), survival percentage (Figure 3), and RWC (Figure 4B) in PC compared with those in WT.

Drought-induced H_2O_2 accumulation in subsidiary cells was shown to be involved in the regulatory signaling of stomatal closure in maize leaves (Yao et al. 2013). Additionally, the activities of antioxidant enzymes were shown to be induced by H_2O_2 in triple *Bermuda* grass, improving its drought resistance (Lu et al. 2009). Inhibition of the oxidative response impaired Cl^- efflux, K^+ efflux, and extracellular alkalinization in tobacco cell suspensions (Cazaléet al. 1998). In the present study, we observed that the H_2O_2 content was all increased in WT and PC suspension cells after 4 and 8 h of the PEG-6000 treatment, but they were still at a lower level in PC than in WT cells (Figure 6B). Phosphoenolpyruvate carboxylase is a cytosolic enzyme in bacteria, cyanobacteria, algae, and higher plants. During C_4 photosynthesis and Crassulacean acid metabolism (CAM), it plays a cardinal role in the initial fixation of atmospheric CO_2 (as HCO_3^-) into the C_4 dicarboxylic acids L-malate and L-aspartate. The low level of H_2O_2 content in PC cells was associated with increased PEPC activity, indicating that H_2O_2 could



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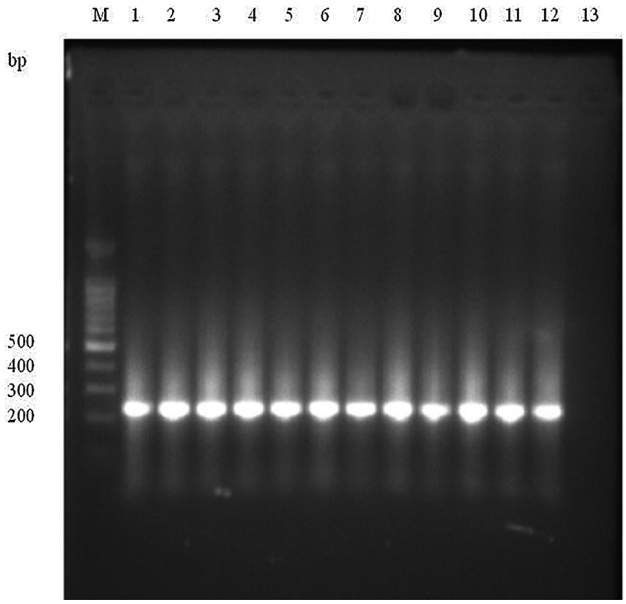

Figure 10. Polymerase chain reaction (PCR) analysis of C_4-*pepc*-transformed (PC) (lane 1-12), wild-type (WT) (lane 13)
M = markers, 1–12 signify the 0.25 kb-amplified product of the C_4-*pepc* gene.

downregulate the PEPC activity in PC under PEG treatment (Figure 6C). The previous research showed that PC can produce more acid metabolites such as oxaloacetic acid and L-aspartic acid due to the high expression of the C_4-*PEPC* gene (Jiao et al. 2005). Therefore, these acid metabolites in PC may counteract the ionic flux caused by PEG stress to alleviate oxidative damage.

The level of NO increases in plants under stress conditions (Cantrel et al. 2011). As shown in Figure 7B, the NO content had significantly increased in both WT and PC suspension cells by 4 and 8 h of PEG-6000 stress. A previous study showed that elevated levels of endogenous NO increased antioxidant enzyme activities, thereby alleviating stress-induced injury in *Poncirus trifoliata* (Fan and Liu 2012). In the present study, addition of cPTIO to the 20% PEG-6000 treatment resulted in a significant increase in PEPC in PC suspension cells. But these changes did not appear in WT in the same treatments (Figure 9D). These results indicated there may be a bypass on regulation of PEPC being independent of NO in PC.

A previous study showed that the free Ca^{2+} content in *Arabidopsis* cells could be enhanced by increases in H_2O_2, which induced expressions of genes encoding antioxidant enzymes (Dodd et al. 2010). Our results showed that, under PEG treatment, the Ca^{2+} content was increased both in WT and PC (Figure 8B). But the level of Ca^{2+} content in PC was much lower than that in WT, and this change of Ca^{2+} content in PC was not consistent with the above findings. The low Ca^{2+} content in PC can induce the increase of SOD after 4 and 8 h of the PEG-6000 treatment (Figure 5B). The magnitude of the Ca^{2+} peak was greater in WT than in PC. Hydrogen peroxide and NO content has no effect on the Ca^{2+} content in PC and WT, suggesting that Ca^{2+} functioned upstream of H_2O_2 and NO in antioxidant defense in plant cells by drought-stress signaling. In PC, by application of EGTA + RR + PEG and EGTA + RR + $La(NO_3)_3$ + PEG, PEPC activity in PC was significantly decreased. These results showed that low Ca^{2+} content reduced the PEPC activity and C_4-*pepc* in PC. The responses of PEPC activity were not observed in WT (Figure 9). This result indicated that Ca^{2+} was involved in signal transduction in the drought-stress response by regulation of PEPC activity and the C_4-*pepc* gene in PC.

Hydrogen peroxide, NO, and Ca^{2+} serve as signaling molecules in plants, regulating different physiological and biochemical processes. They do not function independently, but are interdependent and interactive. Thus, they form unique signal regulatory networks (Li and Xue 2010). During adventitious rooting in the mung bean, H_2O_2 and NO signals showed two parallel pathways, and the Ca^{2+} signal was downstream of both of them (Li and Xue 2010). Lu et al. (2009) found that antioxidant enzyme activities were induced by ABA through H_2O_2 and NO signaling, and that H_2O_2 functioned upstream of NO. As shown in Figure 6, the H_2O_2 content in WT and PC cells under PEG-6000 stress was not affected by addition of cPTIO or EGTA + RR or EGTA + RR + $La(NO_3)_3$. Addition of DMTU or cPTIO or EGTA + RR did not decrease the Ca^{2+} concentration in PC cells at 4 and 8 h of the PEG-6000 treatment (Figure 8). On the contrary, the Ca^{2+} concentration in PC cells, but not WT cells at 4 and 8 h of the PEG-6000 treatment, was obviously decreased by application of EGTA + RR + $La(NO_3)_3$. For NO content, PEG treatment, DMTU + PEG, and EGTA + RR + PEG can increase NO content both in WT and PC. By application of EGTA + RR + $La(NO_3)_3$, NO content in PC cells, but not WT cells at 4 and 8 h of the PEG-6000 treatment, was obviously decreased, suggesting that NO content in PC may be regulated by the calcium influx on the plasma membrane. Based on these results, we speculated that H_2O_2 and Ca^{2+} signaling is involved in the PEPC activity in PC by PEG-6000 treatments and Ca^{2+} is upstream of H_2O_2 in the response to PEG stress in PC.

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Figure 9. Effects of water (CK, not treated), polyethylene glycol (PEG), dimethylthiourea (DMTU), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), ethylene glycol-bis(b-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) + ruthenium red (RR), and EGTA + RR + $La(NO_3)_3$ on phosphoenolpyruvate carboxylase (PEPC) activity in suspension-cultured cells of C_4-*pepc*-transformed (PC) and wild-type (WT) rice
(A) CK, not treated. **(B)** PEG. **(C)** DMTU + PEG. **(D)** cPTIO + PEG. **(E)** EGTA + RR + PEG. **(F)** EGTA + RR + $La(NO_3)_3$ + PEG. Five day old suspension cells were subjected to 20% polyethylene glycol 6000 (PEG-6000) treatments at 28 °C for 8 h. After treating for 0, 4, and 8 h, suspension cells were collected to detect PEPC activity. Error bar represent *SE* and each date represents the mean ± *SE* of three experiments. Bars with different letters were significantly different at the $P < 0.05$ (lowercase) or $P < 0.01$ (uppercase) level according to Duncan's multiple range test.

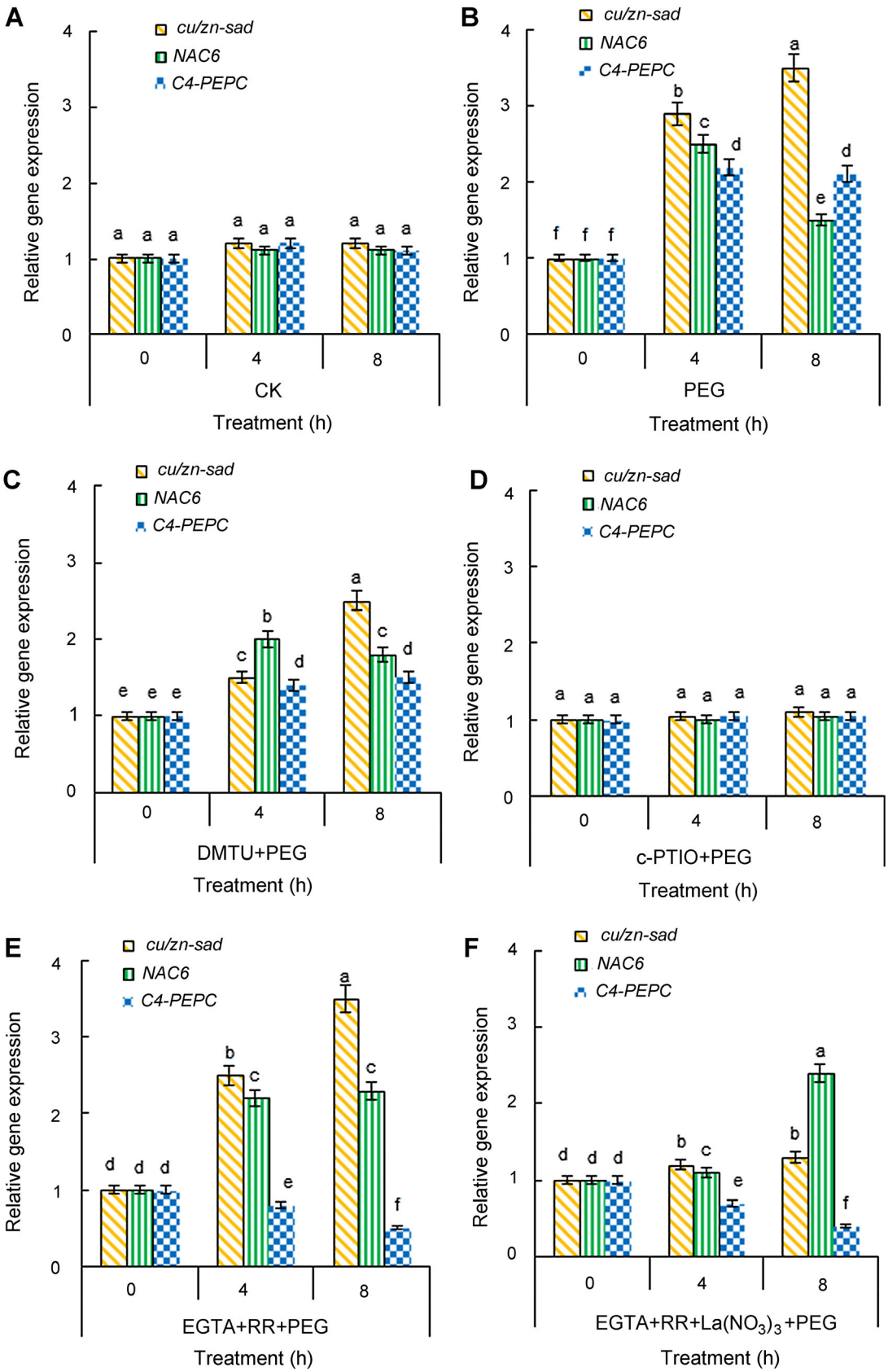

Figure 11. Continued.

Furthermore, when the production of H_2O_2 or NO was inhibited alone, other pathways can also induce the higher PEPC activity in PC. But upon application of EGTA + RR + $La(NO_3)_3$ under PEG treatment, there was a significant decrease in the PEPC activity in PC cells (Figure 9F). These results indicated that the calcium influx on the plasma membrane was the key step for regulation of PEPC activity in PC in the response to PEG-6000 stress. The interactions among these three signaling molecules should be explored in further research.

In addition to abiotic stress, expression of *NAC6* was also changed under these treatments (Figure 11). NAC proteins are plant-specific transcription factors that play essential roles in stress responses. In *OsNAC6*-overexpressing rice, 163 genes were upregulated. These included genes encoding protein kinases, transcription factors, POD, and chitinase (Nakashima et al. 2007). Our qRT-PCR data showed that in PC, *NAC6* transcript levels were significantly enhanced, compared with that in the untreated control under the PEG-6000 treatment. The higher expression of this transcription factor in PC may be related to the increased transcript levels of *C_4-pepc* and *cu/zn-sod*. *C_4-pepc* expression was increased by application of DMTU and EGTA + RR under PEG treatments, suggesting that H_2O_2 and Ca^{2+} were also involved in the regulation of *C_4-pepc* expression. However, for *cu/zn-sod* and *NAC6* gene expression, the application alone with the inhibitor H_2O_2 or NO or calcium had no effect on the expression of the two genes, suggesting that *cu/zn-sod* and *NAC6* may function upstream in the signal regulatory networks in the cells of PC under PEG treatment.

Together, the improved drought tolerance in suspension-cultured cells of PC is associated with elevated PEPC activity and changes in the levels of various signaling molecules under drought stress; in PC, upregulation of Ca^{2+} and down-regulation H_2O_2 induces higher PEPC activity, also enhances upstream gene expression of the transcription factor, such as *NAC6*, and then induces higher downstream gene expression in both *cu/zn-sod* and *C_4-pepc*. These responses in cells of PC enhance the antioxidant system such as SOD and APX activities, which could scavenge ROS, reduce the accumulation of MDA, and allow cells to retain higher RWC and survival percentages for alleviating the drought damage at a cellular level.

MATERIALS AND METHODS

Plant materials

We used the transgenic rice (*Oryza sativa* var. *Japonica* L.) line PC, which overexpresses maize *C_4-pepc*. We used 10th generation plants (Ren et al. 2014), which were derived from third generation plants (Ku et al. 1999). Wild-type rice (*O. sativa* var. *Japonica* L.) plants (cv. Kitaake) were used as the control.

Establishment of rice suspension cell lines

Suspension cell cultures were established as described by Wang et al. (2012a). Suspension cells of rice were cultured under the following conditions: 110 rpm rotation speed, at 28 °C in the dark. Briefly, calluses of uniform size showing normal and healthy growth were selected for use as the experimental materials. The calluses were added to the following medium: N6 basal medium (Chu et al. 1975) containing 2,4-dichlorophenoxyacetic acid (2.4-D) (2 mg/L), sucrose (30 g/L), casein hydrolysate (0.3 g/L), and proline (2.8 g/L), pH 5.8. The liquid medium was refreshed at weekly intervals, and detailed culture protocols of suspension cells were described as our previous methods (Wang et al. 2012a). The synchronous steady cultures were collected separately at the time points indicated, then immediately used in the following experiments.

Application of reagents

The rice suspension cells described above were cultivated in fresh medium for 5 d in logarithmic phase before addition of various reagents or an equal volume of medium (control). The suspension cells were cultured with the various reagents for indicated times. At indicated times during the culture period, and at the end, samples of the cell culture were collected by centrifugation at 1,200 *g* for 10 min. The supernatant and cells were each frozen in liquid nitrogen and stored at −76 °C for further analyses. For the PEG-6000 treatment, suspension cells were cultured in broth containing 20% PEG-6000. Osmotic potential of the solution was 1.01 MPa measured with a freezing point osmometer. Samples were collected as described above at 1, 4, 8, and 12 h, and relevant indicators were measured. The various treatments were as follows: control; 20% PEG-6000; 20% PEG-6000 + 5 mmol/L DMTU; 20% PEG-6000 + 200 μmol/L cPTIO; 20% PEG-6000 + 6 mmol/L EGTA + 0.3 μmol/L RR; and 20% PEG-6000 + 6 mmol/L EGTA + 0.3 μmol/L RR + 25 mmol/L $La(NO_3)_3$ (the pH of stock solution was adjusted to 5.8 with KOH or HCl) (Li et al. 2012). Dimethylthiourea is a H_2O_2 inhibitor (Zhang et al. 2006), cPTIO is an NO synthesis inhibitor (Chen et al. 2014), EGTA is a free calcium chelator, and ruthenium red is a calcium channel outflow inhibitor, and $La(NO_3)_3$ has been described as an inhibitor of Ca^{2+} channels at the plasma membranes (Ding and Pickard 1993). Samples were collected as described above at 0, 4, and 8 h during these treatments, and relevant indicators were measured.

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Figure 11. Effects of water (CK, not treated), polyethylene glycol (PEG), dimethylthiourea (DMTU), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), ethylene glycol-bis(b-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) + ruthenium red (RR), EGTA + RR + $La(NO_3)_3$) on gene expression of *cu/zn-sod*, *NAC6* and *C_4-pepc* in suspension-cultured cells of *C_4-pepc*-transformed (PC) and wild-type (WT) rice

(A) CK, not treated. **(B)** PEG. **(C)** DMTU + PEG. **(D)** cPTIO + PEG. (E) EGTA + RR + PEG. **(F)** EGTA + RR + $La(NO_3)_3$ + PEG. Five day old suspension cells were subjected to 20% polyethylene glycol 6000 (PEG-6000) treatments at 28 °C for 8 h. After treating for 0, 4, and 8 h, suspension cells were collected to detect gene expression of *cu/zn-sod*, *NAC6*, and *C_4-pepc* by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Error bar represent *SE* and each date represents the mean ± *SE* of three experiments. Bars with different letters were significantly different at the $P < 0.05$ (lowercase) or $P < 0.01$ (uppercase) level according to Duncan's multiple range test.

Growth curves of suspension cells and cytological observations

A 20 mL aliquot of suspension cells was added to 80 mL nutrient solution to monitor cell growth. The cell FW and DW with PEG treatment and were determined at 0, 2, 4, 6, 8, 10, and 12 d of growth. Trypan blue staining of cells was conducted as described by Bowling et al. (1997). The suspension cells treated with 20% PEG-6000 were mixed with 0.4% Trypan blue solution at a ratio of 1:9 (final concentration, 0.04%). After 3 min, 10 μL of Trypan blue cell suspension mixture was transferred to a microscopic slide and the viable (unstained) and non-viable (blue stained) cells were counted. For each sample 1,000 cells were scored.

Measurement of MDA content

Malondialdehyde content was determined using the thiobarbituric acid method as described by Dhindsa and Matowe (1981). The absorbance of the reaction mixture was measured at 532 nm.

Measurement of soluble protein content

Soluble protein content was determined using the Bradford (1976) method. Briefly, a 50 μL crude enzyme solution was added to 3 mL G-250 solution, and the absorbance was measured at 595 nm using a UV-1200 ultraviolet spectrophotometer (Meipuda, Shanghai, China). Bovine serum albumin was used as the standard.

Measurement of H_2O_2, NO, and Ca^{2+} contents

The H_2O_2 content was determined as described by Patterson et al. (1984) and Ren et al. (2014). Leaf tissue (0.3 g FW) was homogenized in 3 mL acetone. The homogenate was centrifuged at 10,000 *g* for 10 min at 4 °C, and the supernatant was used for the assay. The supernatant was collected and added to 0.3 mL concentrated hydrochloric acid solution (containing 0.1 mL 20% $TiCl_4$ and 0.2 mL concentrated ammonia). The mixture was incubated at 25 °C for 10 min, and then centrifuged at 8,000 *g* for 10 min at 4 °C. The pellet was washed twice with cold acetone, and then 3 mL 1 mol/L H_2SO_4 was added. The absorbance of the solution was measured at 410 nm, and the amount of H_2O_2 was calculated from a standard curve prepared using known concentrations of H_2O_2.

The NO content in leaves was measured as described by Murphy and Noack (1994). Leaf tissue (0.50 g FW) was incubated with 100 units (U) catalase and 100 U SOD for 5 min to remove endogenous ROS, then 10 mL oxygenated hemoglobin (5 mmol/L) was added and the mixture was incubated for 2 min before measuring absorbance at 550 nm. The amount of NO was determined based on the conversion of oxyhemoglobin into methemoglobin.

The calcium ions (Ca^{2+}) content was measured as described by Yang et al. (1998). Leaf tissue (0.1 g FW) was homogenized in 1 mL extraction buffer (50 mmol/L phosphate buffer, pH 7.4). The homogenate was then centrifuged at 12,000 *g* for 10 min at 4 °C, and the supernatant was collected. The reaction mixture contained 50 μL supernatant, 1 mL methylthymol blue solution (0.25 mol/L methylthymol blue, 0.6% polyvinyl pyrrolidone (PVP), 0.1 mol/L HCl), and 2 mL alkaline solution (0.2 mol/L Na_2SO_3, 0.1% (w/v) glycine, and 0.23 mol/L NaOH). The absorbance of the solution was measured at 610 nm and the amount of Ca^{2+} was calculated from a standard curve prepared using dilutions of a 2.5 mmol/L calcium stock solution.

Measurement of PEPC activity

Phosphoenolpyruvate carboxylase (EC 4.1.1.31) activity was measured as described by Giglioli-Guivarch et al. (1996). Leaf tissue (0.15 g FW) was homogenized in 1.5 mL extraction buffer (50 mmol/L Tris-HCl (pH 7.8), 5 mmol/L dithiothreitol, 1 mmol/L $MgCl_2$, 2% PVP (w/v)) using a mortar and pestle on ice. The homogenate was then centrifuged at 12,000 *g* for 10 min at 4 °C, and the supernatant was used for the assay. The reaction mixture consisted of 700 μL distilled water, 50 mmol/L HEPES-KOH (pH 8.0), 10 mmol/L sodium bicarbonate, 5 mmol/L magnesium chloride, 0.2 mmol/L nicotinamide adenine dinucleotide, 1.5 U malate dehydrogenase, 150 μL crude enzyme solution, and 5 mmol/L PEP. All of the above reagents were added sequentially, and then the mixture was shaken before reading absorbance at 340 nm. The enzyme activity was calculated from the absorbance value.

Measurement of antioxidant enzyme activity

The activities of SOD and CAT were spectrophotometrically measured. Superoxide dismutase and CAT were extracted from approximately 0.5 g (FW) of samples homogenized in 5 mL extraction buffer containing 0.05 mol/L phosphate buffer (pH 7.8) and 1% PVP. The homogenate was then centrifuged at 10,000 *g* for 10 min at 4 °C and the resulting supernatant was used to assay enzyme activities. Superoxide dismutase and CAT activities were measured using the SOD and CAT Detection Kit (A001 and A007; Jiancheng, Nanjing, China) according to the manufacturer's instruction.

Ascorbate peroxidase activity was measured as described by Saruyama and Tanida (1995). The absorbance of the reaction mixture (50 mmol/L phosphate buffer solution (pH 7.0), 0.5 mmol/L ascorbic acid, 0.1 mmol/L ethylenediaminetetraacetic acid, 0.1 mmol/L H_2O_2, and 0.1 mL crude enzyme solution) was measured at 290 nm. One unit of enzyme activity was defined as the amount that caused a 0.1 increase in absorbance per min. The soluble protein content was estimated by the method of Bradford (1976), using bovine serum albumin as the standard.

Measurement of RWC

To measure RWC, 0.5 g suspension-cultured cells per replicate under different treatments were obtained from the central third of leaves using a circular cutter. The cells were weighed to obtain FW, and then floated on water at 4 °C in the dark. After 18 h, the turgid weight (TW) was measured. Cells were then dried at 75 °C to constant weight and DW was obtained. The RWC (%) was calculated as follows:

$$RWC = (FW - DW)/(TW - DW) \times 100$$ (Anjum et al. 2011).

Extraction of total RNA, semiquantitative PCR, and qRT-PCR

Total RNA was extracted using an RNA simple Total RNA Kit (Tiangen, Beijing, China), according to the manufacturer's instructions. Reverse transcription reactions were conducted using a TaKaRa PrimeScript RT Master Mix Perfect Real Time Kit (TaKaRa, Dalian, China) and qRT-PCR analyses were conducted using an SYBR Premix Ex TaqTM II kit (TaKaRa, Dalian, China), according to the manufacturers' instructions.

We used Applied Biosystems instruments (Foster City, CA, USA) for these analyses. Semiquantitative PCR was conducted as described by Dubouzet et al. (2003) using the following gene primers: *pepc*, 5′-CATCCTGCACATGCTCAACC-3′ (forward), 5′-ACGCCCTTCCATACAGTCTC-3′ (reverse); *cu/zn-sod*, 5′-CTGGGCCACACTACAATCCT-3′ (forward), 5′-CAGCCTTGAAGTCCGATGAT-3′ (reverse); *NAC6*, 5′-CGCTGTACGGAGAGAAGGAG-3′ (forward), 5′-ACTCGTGCATGATCCAGTTG-3′ (reverse); and *actin*, 5′-CCCTCTTTCATCGGTATGGA-3′ (forward), 5′-TTGATCTTCATGCTGCTTGG-3′ (reverse).

Statistical analysis

The data were analyzed by one-way ANOVA and least significant difference multiple comparison tests ($P < 0.05$) using SPSS version 18.0 software (SPSS, Chicago, IL, USA). We analyzed qRT-PCR data using the $2^{-\Delta\Delta Ct}$ method.

ACKNOWLEDGEMENTS

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