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Full Length Research Paper

Effects of 1-butanol, neomycin and calcium on the photosynthetic characteristics of *pepc* transgenic rice

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The effects of 1-butanol, neomycin and calcium on the photosynthesis of *pepc* transgenic rice were studied with over-expressed *pepc* transgenic rice (PC) of the 8th generation as study materials and with non-transgenic wild type (WT) rice and maize, a typical C_4 plant, as control. The results show that, 0.04% 1-butanol and 0.05 mmol L^{-1} Ca^{2+} has a significant effect on the net photosynthetic rate (P_n) of the three tested materials. Furthermore, when compared with the WT, PC under different treatments maintained a relatively high P_n. The stepwise multiple linear regression analysis showed that, PC maintained its relative high P_n through the increase of stomatal conductance. It was proved by the scanning electron microscope (SEM) that, when compared with the WT, the stomatal density of PC leaves increased while the stomatal aperture also increased under the 1-butanol treatment. The phosphoenolpyruvate carboxylase (PEPC) activity of PC and maize leaves were significantly inhibited after the 1-butanol treatment alone, while they had no significant effects after the neomycin treatment alone. However, the combined treatment with 1-butanol and neomycin evidently promoted the PEPC activity of PC and WT. Moreover, exogenous calcium significantly promoted the PEPC activity of the three materials. It is clear that, the exogenous regulation on PC is different from both C_4 and C_3 plants. PC might regulate stomatal aperture primarily by the calcium-mediated phospholipase D (PLD) pathway, which also increases the leaf stomatal density, thus, raising the stomatal conductance and showing a relatively high P_n.

Key words: *Pepc* transgenic rice, 1-butanol, neomycin, photosynthesis, stomatal conductance.

INTRODUCTION

The successful transfer of the phosphoenolpyruvate carboxylase (*pepc*) gene from C_4 maize into rice and its over-expression clearly promote rice photosynthesis, which has triggered an upsurge of strengthening C_4 photosynthetic characteristics in C_3 plants in order to enhance its photosynthetic efficiency. This is of great significance to raise rice production by increasing the photosynthetic capacity (Ku et al., 1999; Fukayama et al., 2002). In recent years, with the stable breeding of the transgenic rice over-expressing maize *pepc*, an increasing number of studies have demonstrated that, it has a high photosynthetic efficiency and various stress resistances against oxidation, drought, etc (Jiao et al., 2001; Huang et al., 2002; Jiao et al., 2003; Li et al., 2005; Zhang et al., 2006; Zhang et al., 2007). The way that a single transferred C_4 *pepc* gene drives this complex photosynthesis of C_3 rice is an interesting scientific topic. When studying phosphatidic acid (PA) in plants, Christa (2004) found that, *pepc* could act as the target of PA and take precedence over other phospholipids in combination with PA. PA as an inositol-dependent metabolism regulator has certain functions in the signaling transduction pathway (Loewen et al., 2004). PA is an important lipid signal molecule which can be produced by two

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Abbreviations: PEPC, Phosphoenolpyruvate carboxylase; **PC,** *pepc* transgenic rice; **P_n,** net photosynthetic rate; **PLD,** phospholipase D**; PLC,** phospholipase C; **PA,** phosphatidic acid; **SEM,** scanning electron microscope; **NC,** nitrocellulose membrane.

different pathways, namely, phospholipase D (PLD) and phospholipase C (PLC) pathways. These two pathways may exist in different sub-cellular sites and start from different phosphate precursors, showing different specific activity towards downstream targets (Munnik et al., 2004; Testerink and Munnik, 2005). Moreover, the phosphoinositide pathway and intracellular calcium may play an important regulatory role in the light-dependent phosphorylation of C_4 *pepc* (Coursol et al., 2000; Giglioli et al., 1996; Gousset et al., 2005; Jose et al., 2007, Li et al., 2010).

PA can regulate cell processes in different modes and perform various biological functions at different times, spaces and concentrations (Wang et al., 2006). Although, phosphoenolpyruvate carboxylase (PEPC) has been proved to act as a target of PA, its mechanism remains unclear. With *pepc* transgenic rice as a unique material, the study of impact of PA on its photosynthetic characteristics may be helpful to reveal the regulatory characteristics of PA signaling pathway towards the exogenous PEPC of *pepc* transgenic rice. In this paper, PLD specific inhibitor, 1-butanol (Munnik et al., 2001) and PLC specific inhibitor, neomycin (Van et al., 1988), were used to inhibit respectively the production of PA from different sources, while calcium was applied to regulate the PEPC phosphorylation state, through which the impact of the three materials on the photosynthetic characteristics of *pepc* transgenic rice was studied in the overall plant level, photosynthetic sub-processes, enzymology, genetic level, etc, in hope of finding the way to regulate the C_4 *pepc* transgenic rice to enhance the photosynthetic rate via the PA signaling pathway .

MATERIALS AND METHODS

A stable *pepc* transgenic rice (PC) of the 8th generation (*Oryza sativa* L.), wild type rice, Kitaake (WT) (*O. sativa* L.) and maize, Nongda 108 (*Zea mays* L.), was used in this study. The experiment took place in the greenhouse of Jiangsu Academy of Agricultural Sciences in 2007 and 2008. In late April, rice seeds were soaked for 24 h after disinfected by 5% H_2O_2 for 5 min. Then the seeds were stored at 30°C for 48 h for pregermination and sown on May 1st. On May 15th, the rice seedlings were transplanted into pots, 5 holes per pot, 1 seedling per hole via the randomized block design. At the same time, maize was planted in the field with regular management of water and fertilizer.

Application of reagents

The PC blossomed after four to seven days with the flag leaf of WT and the last leaf (first) under tassel of maize was collected for indices measurement during 9:00 to 11:00 am on a sunny summer day in Nanjing, with the light intensity of (1200 ± 100 μmol m^{-2} s^{-1}) and temperature (33 ± 2°C). The treatment solutions containing 1-butanol, neomycin and calcium chloride were absorbed by the leaf sheath with the concentration 0.04%, 0.1 mmol L^{-1} and 0.05 mmol L^{-1}, respectively. The materials for the measurement of photosynthesis indices and various fluorescence physiological indices were treated as follows: the bottom leaf sheath of rice and the base of maize leaf were cut and immediately placed in distilled water and the leaf sheath was cut at its lower part to remove air bubbles in the vascular bundles, so as to measure photosynthesis indices and various fluorescence physiological indices. Then, the materials were immediately put into the treatment solutions. The lower part of the leaf sheath was cut again and placed under natural light and temperature for 30 min before photosynthesis indices and various fluorescence physiological indices were re-measured. The materials for the measurement of protein contents and gene expression levels were treated as follows: The leaf was divided into two parts through the leaf vein and the non-vein part of the leaf was immediately clipped off as the sample, wrapped in the foil and stored in liquid nitrogen, the leaf sheath at the bottom of this rice leaf and the base of maize leave were cut immediately and placed into the treatment solutions, while the lower part of the sheath was cut out and placed under the natural light and temperature for 30 min, then, the other half of the leaf was clipped off, wrapped in foil and immediately stored in liquid nitrogen for further measurement.

Measurement of net photosynthetic rate

The LI-6400 portable photosynthesis analyzer produced by the LI-COR Inc., United States, was used in the measurement via the red and blue light source under the open system, with the following conditions: 1200 μmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD), 400 μmol s^{-1} flow rate and the leaf chamber temperature of 25°C. In each treatment, first leaves under tassel (last three) were adopted for measurement and each leaf was measured for four to six times.

Measurement of chlorophyll fluorescence parameters

The kinetic parameters of chlorophyll fluorescence were measured according to Genty et al. (1989) with the FMS2 fluorescence analyzer (Hansatech, UK) under the conditions of 4000 μmol m^{-2} s^{-1} saturation pulse light and 0.9 μmol m^{-2} s^{-1} flashes with an interval of 30 s. The leaf was treated by dark adaptation for 15 min before chlorophyll fluorescence measurement. Then the initial fluorescence (F_o) and the maximum fluorescence (F_m) were directly read out to calculate the variable fluorescence F_v through the formula $F_v = F_m - F_o$, thus, the primary light energy conversion efficiency of PS II was obtained via F_v/F_m. In each treatment, first leaves under the tassel (last three) were adopted for measurement and each leaf was measured for three times.

Measurement of PEPC enzyme activity

PEPC activity of the experimental materials was measured according to Gonzalez et al. (1984) with the treatment solutions of 0.04% 1-butanol, 0.1 mmol L^{-1} of neomycin, 0.05 mmol L^{-1} of calcium and 0.2 mmol L^{-1} ethylene glycol tetraacetic acid (EGTA), respectively.

Protein extraction and electrophoresis

Leaves (0.5 g) were harvested and ground in extraction medium (50 mmolL^{-1} Tris-HCl, pH 7.5 containing 1 mmolL^{-1} $MgCl_2$, 5 mmolL^{-1} DTT and 2% (w/v) insoluble PVP). After complete maceration, the crude extract was centrifuged at 13000 g for 10 min and proteins were extracted from the supernatant. The total protein contents of PEPC crude extracts mentioned earlier were measured according to the method of Bradford (1976). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis was conducted with 10 mg protein sample per well at temperature

(4℃) and constant voltage (120 V) for 2 h.

Western blot analysis

The gel was soaked in transfer buffer (150 mmolL^{-1} glycine, 20 mmolL^{-1} Tris-HCl, 0.01% sds, 4% methanol, pH 8.8). A nitrocellulose membrane (NC) was then applied and the setup was electrophoresed at 250 mA for 1.5 h, in order to transfer the protein onto the NC. The transferred NC was soaked in blocking buffer (1% nonfat milk powder, 20 mmolL^{-1} Tris-HCl, pH 8.0,100 mmolL^{-1} NaCl) at room temperature for 2 h before addition of the antibody (anti-PEPC) and incubated at room temperature for 1 h. Following this incubation, the NC was washed by PBS (3×, 10 min each) and goat anti-rabbit Ig G (1:1500) were added to the blocking buffer for 1 h. Then, the NC was washed with TBS (20 mmolL^{-1} Tris-HCl, pH 8.0,100 mmol/L NaCl; 3× for 10 min each). Finally, the NC was developed using AP (100 mmolL^{-1} Tris-HCl, pH 8.0,100 mmolL^{-1} NaCl, 5 mmolL^{-1} $MgCl_2$, BCIP/NBT).

Gene transcription of PEPC

A rapid extraction kit for total RNA with high purity (centrifugal columnar type) and a cDNA synthesis kit for M-MLV first chain (Shanghai Jierui Bio-Engineering Co., Ltd.) were used in the total RNA extraction of treated leaves in accordance with procedures in order to transform RNA into first chain cDNA that was used as the RT-PCR template. A pair of primers specific to the cDNA of C_4 PEPC were designed via BLAST, namely, the upstream primer 5'-catcctgcacatgctcaacc-3' and downstream primer 5'-acgccctt-ccatacagtctc-3' synthesized by Shanghai Jierui Bio-Engineering Co., Ltd., in order to amplify a 465 bp cDNA fragment. With gene *Racl of Actin* constitutively expressed in rice as the internal reference, the PCR primers of *Racl* gene, 5'-ggaactggtatggtcaaggc-3 'and 5'-agtctcatggataaccacag-3', were amplified. The PCR amplification procedures were as follows: pre-denaturation was carried out at 94℃ for 5 min, denaturation at 94℃ for 40 s, annealing at 55℃ for 40 s and extension at 72℃ for 1 min, with a total of 28 cycles. At last, the sample was kept in 72℃ for 10 min. Reaction products were separated by electrophoresis in 2% agarose gel at 120 V for 40 min. Then, the result was imaged by BIO-RAD quantity one, a gel imaging system for observation and photographic archive.

Electron microscope preparation

Rice materials were cultivated in a tissue culture room via sand culture under the conditions of 16 h light/ 8 h darkness, light intensity of 200 µmol m^{-2} s^{-1} and 25℃. Other conditions were regularly managed according to the laboratory conditions. PC and WT materials of relatively the same size were chosen and divided into groups, three plants per group. The samples were respectively treated according to the leaf sheath absorption method mentioned earlier with the light intensity of 1000 µmol m^{-2} s^{-1} through treatment solutions of 0.04% 1-butanol, 0.1 mmol L^{-1} neomycin and 0.05 mmol L^{-1} calcium chloride. The leaves were placed perpendicular to the light source and treated by scanning electron microscopy for 30 min. Three last second leaves (the second fully expanding leaves) were observed by electron microscopy in each treatment.

Leaf observation via scanning electron microscopy (SEM)

The materials were immediately put into pre-cooled 2.5% (pH 7.2) glutaraldehyde after separation for air extraction and fixation for 6 to 8 h, rinsed with distilled water and dried with tert-butanol. The well dried samples were fixed and coated with their observation side upward. Then, they were placed on a Hitachi S-3000N scanning electron microscopy to be observed, photographed and recorded.

Data analysis

The disparity analysis was conducted with statistical software SPSS13.0. The stepwise multiple linear regression analysis of the material photosynthetic parameters was carried out with DPS statistical software. The quantitative analysis of the SDS-PAGE and RT-PCR bands was obtained via Band Scan5.0 and Band leader, respectively. Data were processed and mapped with Excel 2003.

RESULTS

Effects of 1-butanol, neomycin and calcium on the photosynthetic rate of the materials

Figure 1 indicates the net photosynthetic rate (Pn) of PC flag leaf was significantly higher than that of WT with a significant difference ($P < 0.05$). Furthermore, there was a significant difference ($P < 0.05$) in terms of Pn between three materials treated with 1-butanol, neomycin and calcium ions and the untreated control group. With 1-butanol, the percentages of Pn between before and after treatments were 54.90% of PC, 97.96% of WT and 72.26% of maize; with neomycin, others were 95.23% of PC, 91.13% of WT and 98.30% of maize; with calcium ions, others were 113.62% of PC, 109.55% of WT and 108.70% of maize. Interestingly, the treatment solutions had no evident effects on intercellular CO_2 concentration (Figure 3), but significantly different in stomatal conductance (Figure 2).

The stepwise multiple linear regression analysis further indicated that, after 1-butanol, neomycin and calcium ion treatments, the regression equation coefficients of stomatal conductance were 16.589, 5.456 and 21.309, respectively, which were the largest regression parameters of each equation. It demonstrated that, PC maintained a high net photosynthetic rate mainly by increasing stomatal conductance under different treatments.

However, through the SEM observation, the average stomatal density was 225 mm^{-2} on the back of PC leaves, significantly higher than that of WT with 185 mm^{-2} (Table 1), showing that, the high photosynthetic capacity of PC was not only related to CO_2 concentrating mechanism in PC (Jiao et al., 2003), but also to its stomatal density, which was different from the previous studies (Huang et al., 2002). Interestingly, it was found by the SEM (Figure 4) that, after 1-butanol treatment for 30 min, PC leaves increased stomatal aperture, stomatal width extending from 1.51 to 2.12 µm ($P < 0.05$), while there was no significant difference in that of WT (Table 1). It seems that the regulation of high photosynthetic efficiency in PC may be involved in the inositol phosphate signaling pathways of PLD mediated by PA, so as to regulate stomatal movement.

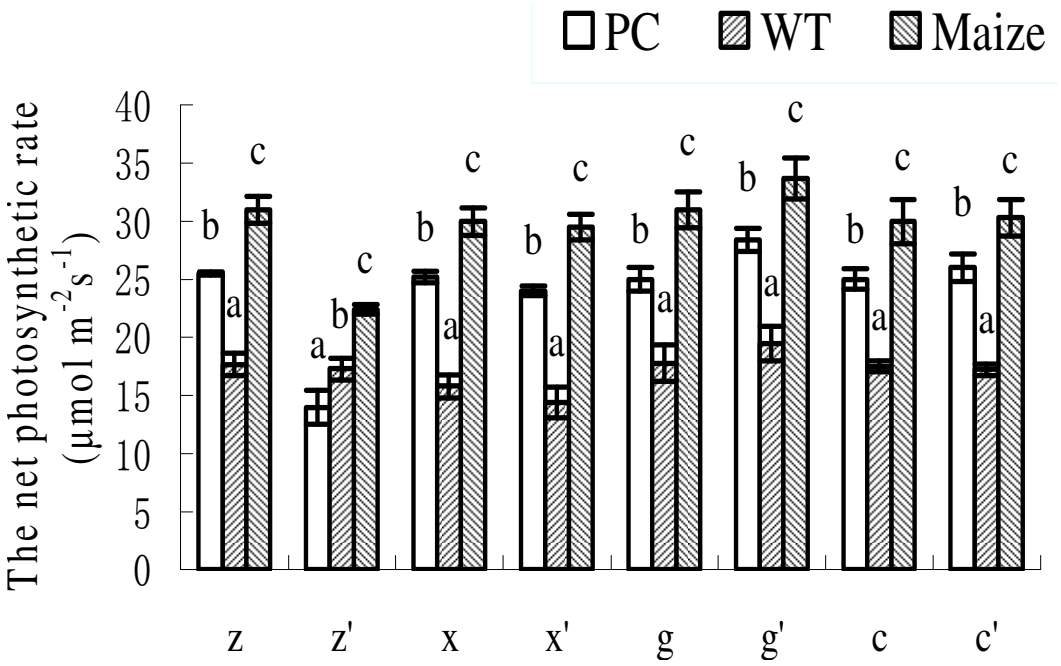

Figure 1. Effects of 1-butanol, neomycin and calcium on photosynthesis of the PC, WT and maize. Values followed by a different letter are significantly different at $P < 0.05$. z, before 1-butanol treatment; z', after 1-butanol treatment; x, before neomycin treatment; x', after neomycin treatment; g, before calcium treatment; g', after calcium treatment; c, before control treatment; c', after control treatment. WT, wild type; PC, *pepc* transgenic rice.

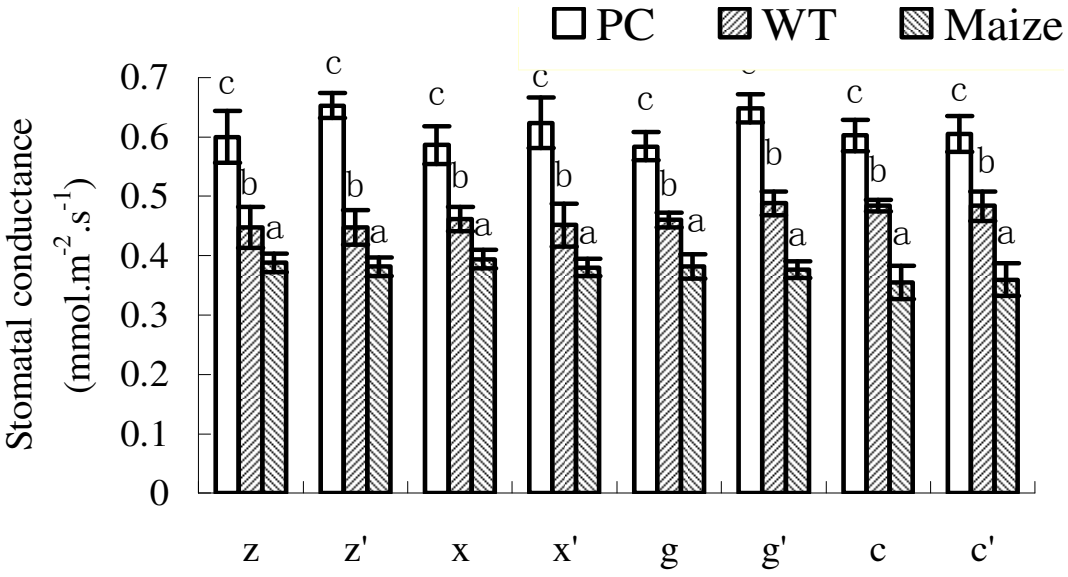

Figure 2. Effects of 1-butanol, neomycin and calcium on stomata conductance of leaf of the PC, WT and maize. Values followed by a different letter are significantly different at $P < 0.05$. WT, wild type; PC, *pepc* transgenic rice. Treatments are described as in Figure 1.

Effects of 1-butanol, neomycin and calcium treatments on fluorescence parameters of various materials

The effects of 1-butanol, neomycin and calcium on chlorophyll fluorescence parameters of the three materials further reflected their impacts on the primary reaction of photosynthesis (Figure 5). The results indicate that, 1-butanol treatment significantly reduced the primary light energy conversion efficiency F_v/F_m ($P < 0.05$) in PS II

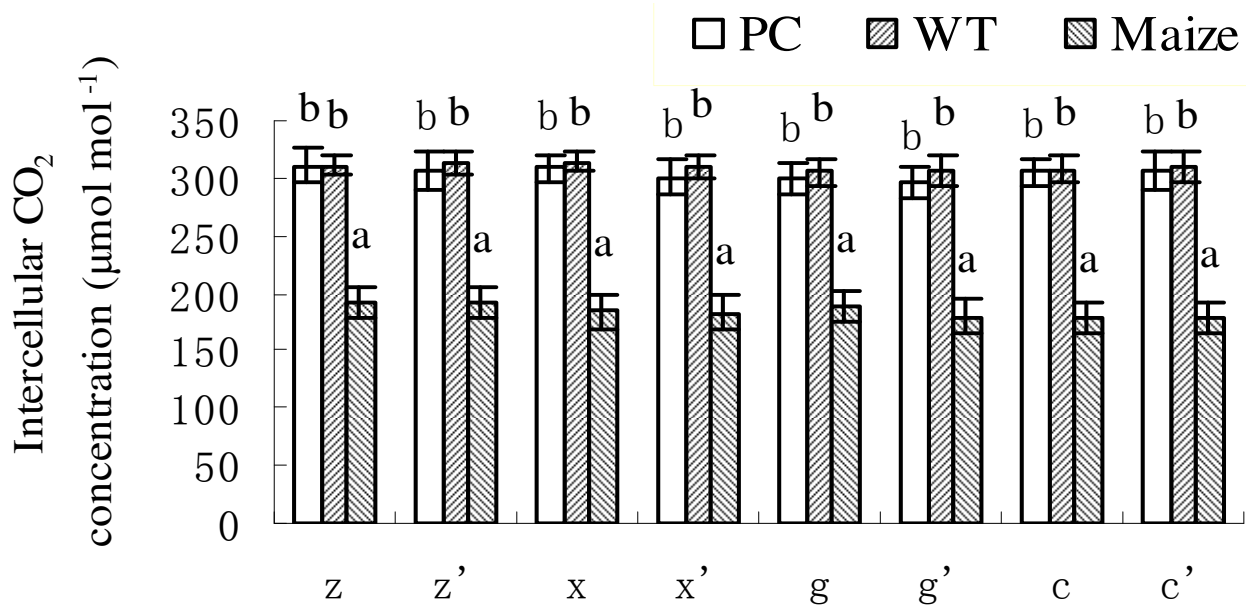

Figure 3. Effects of 1-butanol, neomycin and calcium on intercellular CO_2 concentration of leaf of the PC, WT and maize. Values followed by a different letter are significantly different at $P < 0.05$. WT, wild type; PC, *pepc* transgenic rice. Treatments are described as in Figure 1.

Table 1. Effects of 1-butanol on stomata density, stomata length and stomata width of the back of PC and WT leaves.

Type of rice	Treatment	Stomata density (mm^2)	Stomata length (μm)	Stomata width (μm)
PC	Before 1-butanol treatment	225 ± 5	15.56 ± 0.28[a]	1.51 ± 0.14[b]
	After 1-butanol treatment		15.32 ± 0.14[a]	2.12±0.24[c]
WT	Before 1-butanol treatment	185 ± 7	15.64 ± 0.20[a]	0.93 ± 0.11[a]
	After 1-butanol treatment		15.70 ± 0.34[a]	0.92 ± 0.09[a]

WT, wild type; PC, *pepc* transgenic rice. Specify the significance for the superscript.

of WT. However, its effect on the F_v/F_m of PC and maize was not significant, indicating that 1-butanol had little impact on the light energy conversion sub-process in PC, similar to that of maize.

Effects of 1-butanol, neomycin and calcium on the enzyme activity of PEPC

The effects of 1-butanol, neomycin and calcium on the PEPC activity of the tested materials showed (Figure 6) that, 1-butanol inhibited the PEPC activity of PC and maize by 25.7 and 10.2%, respectively (Figures 6a to c), but promoted PEPC activity of WT by 16.8% (Figure 6b), all in significant differences ($P < 0.05$); neomycin had no significant effect on PC and maize, but it resulted in an increase of 17.9% in PEPC activity of WT ($P < 0.05$); calcium cleared with EGTA in cells significantly inhibited the PEPC activity of the three materials, among which the effect on PC was the most significant with a percentage of 22.9%. Calcium could also clearly promote PEPC activity of the three materials when added alone. The PEPC activity of PC was promoted by 66.8%, the most significant among the three materials ($P < 0.05$). The results indicate that, the trend of the effects on the exogenous PEPC activity of PC was similar to that of maize when 1-butanol and neomycin were added separately, but the effect on PC was significantly stronger than that of maize.

At the same time, calcium also promoted the PEPC activity of PC and WT after they had been treated by the combined solution of 1-butanol and neomycin, registering an increase of 32.4% in PEPC activity of PC, higher than the result of the treatment with calcium.

This treatment also offset the 1-butanol inhibition of PEPC activity.

However, the PEPC activity of maize under this treatment was decreased, showing that change of exogenous PEPC enzyme in PC was markedly different from that of maize under this condition.

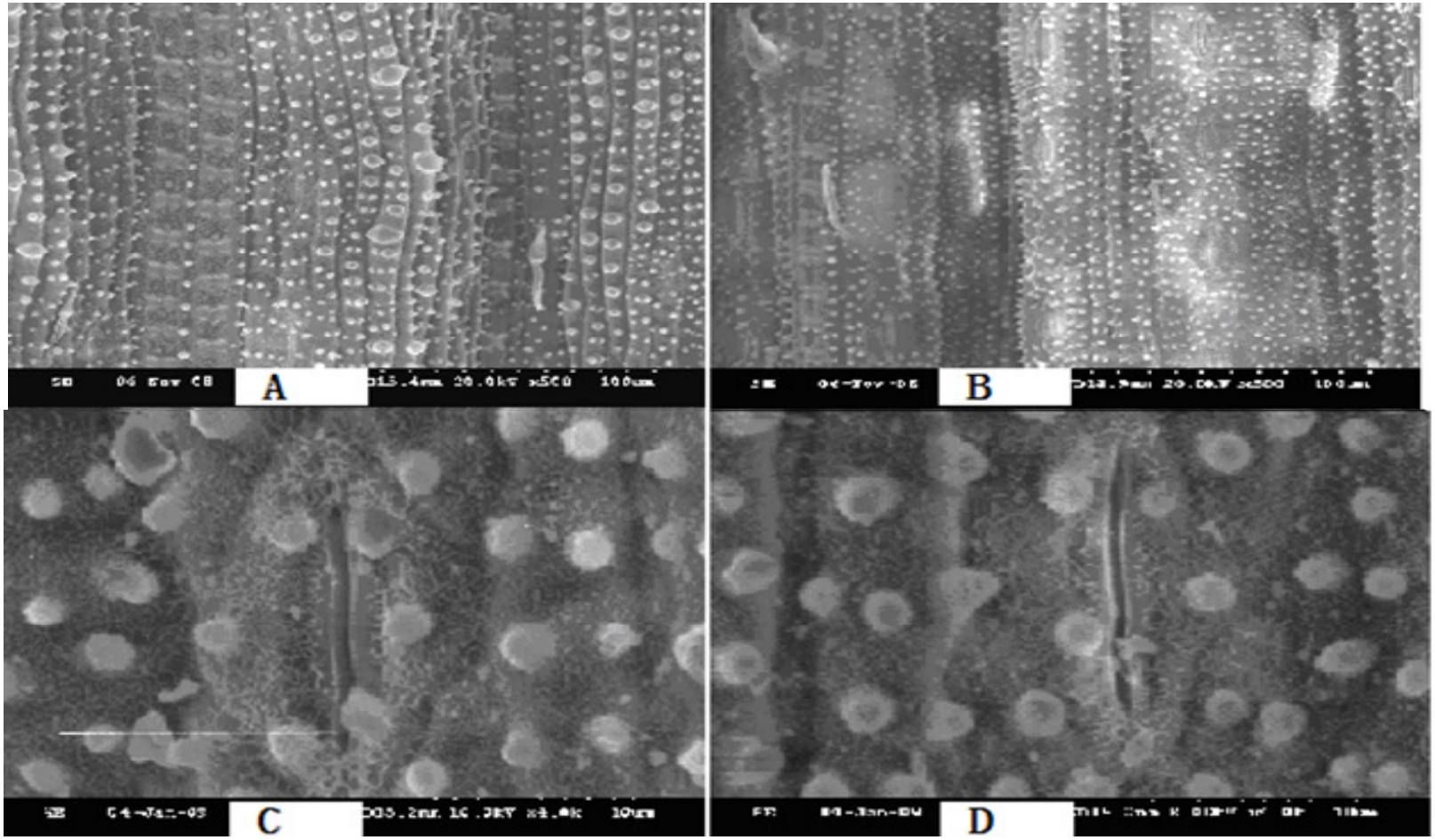

Figure 4. Effects of 1-butanol on stoma of the back of PC and WT leaves. A, the back of the structure of WT leaves; B, the back of the structure of PC leaves; C, the stoma of the back of WT leaf under normal situation; D, the stoma of the back of WT leaf under treatment of 1-butanol; E, the stoma of the back of PC leaf under normal situation; F, the stoma of the back of PC leaf under treatment of 1-butanol.

Effects of 1-butanol, neomycin and calcium on *pepc* gene transcription and protein expression of all materials

1-butanol, neomycin and calcium affected the activity of PEPC, but whether it was caused by the impact transcription or translation of *pepc* gene remained unclear. The statistical analysis of western blot and RT-PCR bands showed that, PEPC protein and gene expression did not change significantly after the 1-butanol, neomycin and calcium treatments of the three materials (Figures 7 and 8). However, when compared with untreated controls, the gene expression of the materials changed after their treatment with 1-butanol and neomycin, while the trend of the gene expression after calcium treatment was in line with that of the controls. Figure 7 indicates that, the C_4

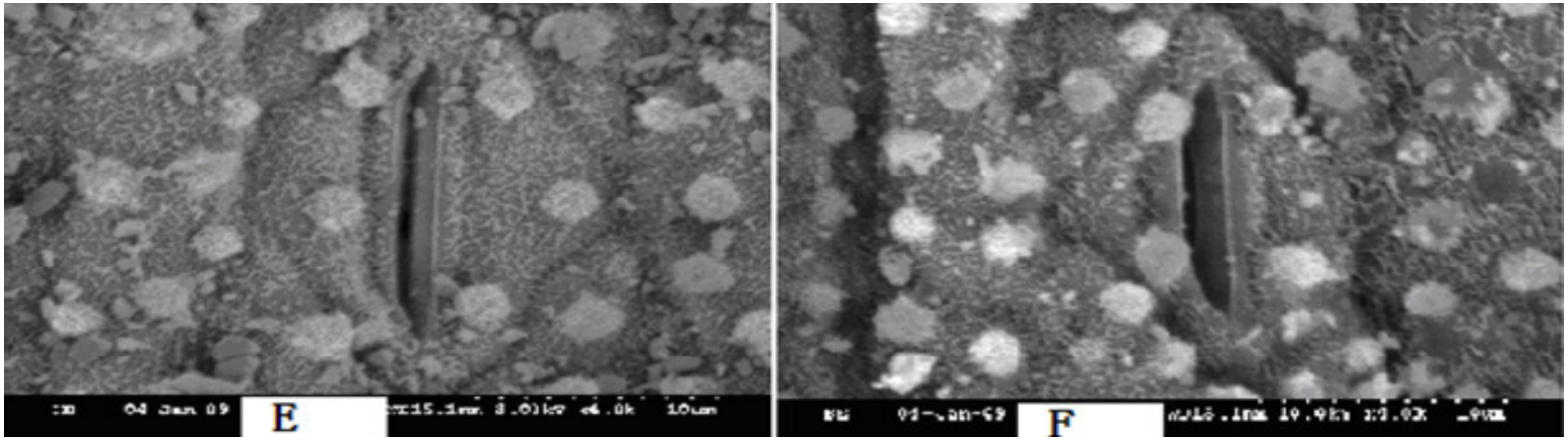

Figure 4. Contd.

pepc gene transferred from rice achieved high protein expression, the C_3 PEPC showed little expression in the WT (Figure 7) and at the same time, the primers of C_4 *pepc* gene also failed to amply its band, demonstrating that the interesting effects of different chemical agents on PC may be related to its gene expression.

DISCUSSION

In conditions such as strong light, high temperatures and drought, C_4 plants have a clear advantage in growth, possessing higher water content, rate of nutrient utilization and biological yield (Aoyaki and Bassham, 1986). Thus, many efforts have long been taken to introduce some photosynthetic characteristics of C_4 plants into C_3 plants, so as to increase their photosynthetic efficiency and to gain resilient C_3 plants with high yields. However, there has been no substantial progress via conventional hybridization method (Surridge et al., 2002). In recent years, because of the cloning of C_4 photosynthesis enzyme gene (Ku et al., 1996 Matsuaka; 2001) and gene transfer, the genetic limitations among plant species have been broken, thus, a series of transgenic C_3 plants with PEPC, pyruvate orthophosphate dikinas (PPDK) and NAD (P)-dependent malic enzyme (NAD (P)-ME) of C_4 plants have been obtained (Fukayama et al., 2003; Chi and Zhang, 2004; Liu et al., 2005; Takeuchi et al., 2000; Bandyopadhyay et al., 2007; Zhang et al., 2007). Moreover, many transgenic plants with C_4 photosynthesis gene have changed in physiological characteristics, such as the increase of photosynthetic rate (Liu et al., 2005; Takeuchi et al., 2000; Bandyopadhyay et al., 2007), the reduction of oxygen inhibiting photosynthesis (Ku et al., 1999), resistance against heavy metals such as aluminum (Sheehy et al., 2000). The transgenic rice with entire *pepc* gene from maize not only has significantly improved photosynthetic efficiency, but also raised the grain yield (Sheehy et al., 2000).

C_4 *pepc* is the key gene to improve the photosynthetic efficiency in rice (Jiao et al., 2001). With high light intensity and temperature, the high photosynthetic efficiency of C_4 *pepc* transgenic rice is based on the induction of C_4 photosynthetic enzymes in leaves and on the increase of carbonic anhydrase activity involved in CO_2 transportation, which increases CO_2 concentration in the vicinity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and decreases CO_2 intercellular leakage in leaves, thus, bringing about primary CO_2 concentrating mechanism (Long et al., 2006). The mere introduction of C_4 *pepc* gene into rice can give rise to the knock-on effects in the structure of photosynthetic pathways as well as matter, energy and other multi-metabolic pathways in rice (Jiao et al., 2002, 2003; Li et al., 2007; Yuan et al., 2006; Wang and Li,2007), which is a scientific issue deserving further exploration. Recent research also indicated that, PC under strong light can keep a steady photosynthetic structure to avoid the damage from strong light, which might be related with PLD/PA signaling pathway (Li et al., 2010). In this paper, through the introduction of exogenous 1-butanol and neomycin, specific PA inhibitors

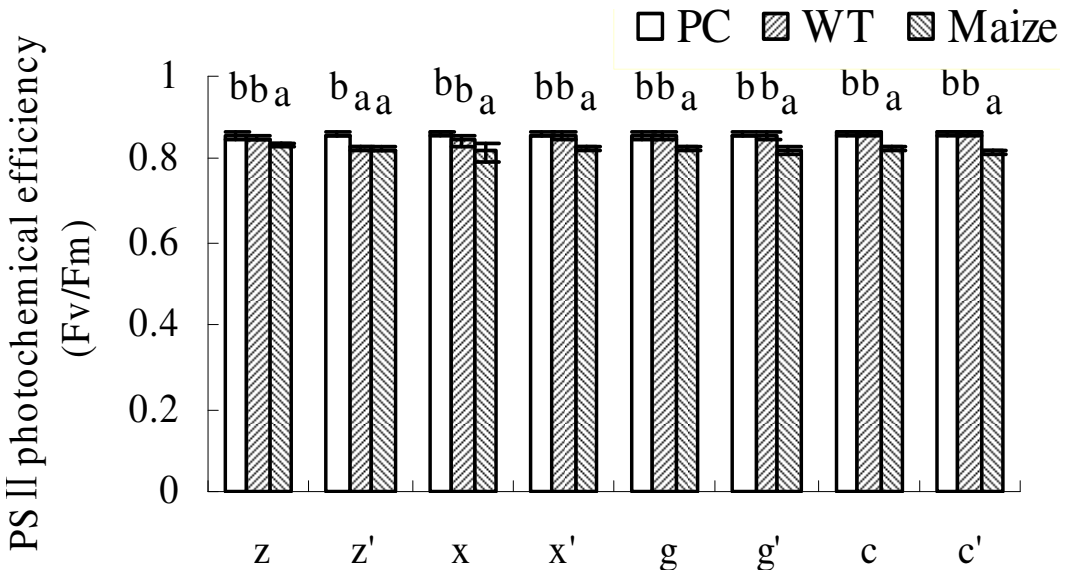

Figure 5. Effects of 1-butanol, neomycin and calcium on Fv/Fm of the PC, WT and maize. Values followed by a different letter are significantly different at $P < 0.05$. WT, wild type; PC, *pepc* transgenic rice. Treatments are described as in Figure 1.

in the inositol phosphatesignaling pathway, it is found that PC regulates the PEPC activity mainly through the phospholipase PLD pathway. Moreover, it is also found that, 1-butanol can induce the increase of stomatal aperture in leaves. Regulating stomatal opening in PC, the 1-butanol is also a negative factor that significantly inhibits exogenous PEPC activity.

These regulatory features are related to gene expression and protein translation to a certain extent, indicating that the high photosynthetic characteristics of PC may be closely related to the regulators of PEPC activity and stomatal aperture. On one hand, this high photosynthetic efficiency may be related to the structural changes by increasing stomatal density in leaves. On the other hand, it is also related to the PLD signaling pathway which plays a role in the gene regulation, molecular regulation of PEPC activity and stomatal aperture control. The combination of over-expressed PEPC enzyme and PA appropriately decreases the stomatal aperture to reduce intracellular CO_2 leakage so as to increase photosynthetic substrate and adapt to environmental changes. This is in line with the physiological characteristics of resistance to photoinhibition and drought of this material (Jiao et al., 2001; Huang et al., 2002; Wang et al., 2002; Jiao et al., 2003; Li et al., 2001, 2005).

Exogenous calcium, a regulator of stomatal closure can also significantly improve the exogenous PEPC activity in PC and reverse through calcium removal agent. EGTA indicating that, the molecular regulation of exogenous PEPC enzymes in PC depends on the calcium. It is noteworthy that, when the PLC and PLD pathways are inhibited, the PEPC enzyme activity in PC is induced to increase and such inducement is strengthened by exogenous calcium ions, which is significantly different from the PEPC enzyme changes in maize. Presumably, when the synthesis of PA is inhibited, PC may rely on other calcium-related ways to compensate for it. This shows that, the regulation process of PEPC enzyme in PC is different from a typical C_3 plant, WT and a typical C_4 plant (maize).

But the regulatory process also needs calcium. When combined with the effects on PC photosynthetic parameters and stomatal density, it is clear that PC increases its stomatal conductance through raising its stomatal density.

However, because of the lack of vascular bundle sheath cells in typical C_4 plants, PC appropriately reduces the aperture in each stomatal to decrease the leakage of intercellular CO_2 concentration in order to adapt to environmental pressure, thereby enhancing the photosynthesis substrate and its photosynthetic rate. The research on how the signal transduction via PA in PEPC transgenic rice need further studied.

Conclusions

Structurally, *pepc* transgenic rice enhances stomatal conductance by increasing the leaf stomatal density, thus, affecting its photosynthetic characteristics. For the regulation of exogenous PEPC activity, PA is needed to function as the second intracellular signaling molecule and interact with PLD or PLC process with the participation of calcium, thus, maintaining its high photo-synthetic capacity.

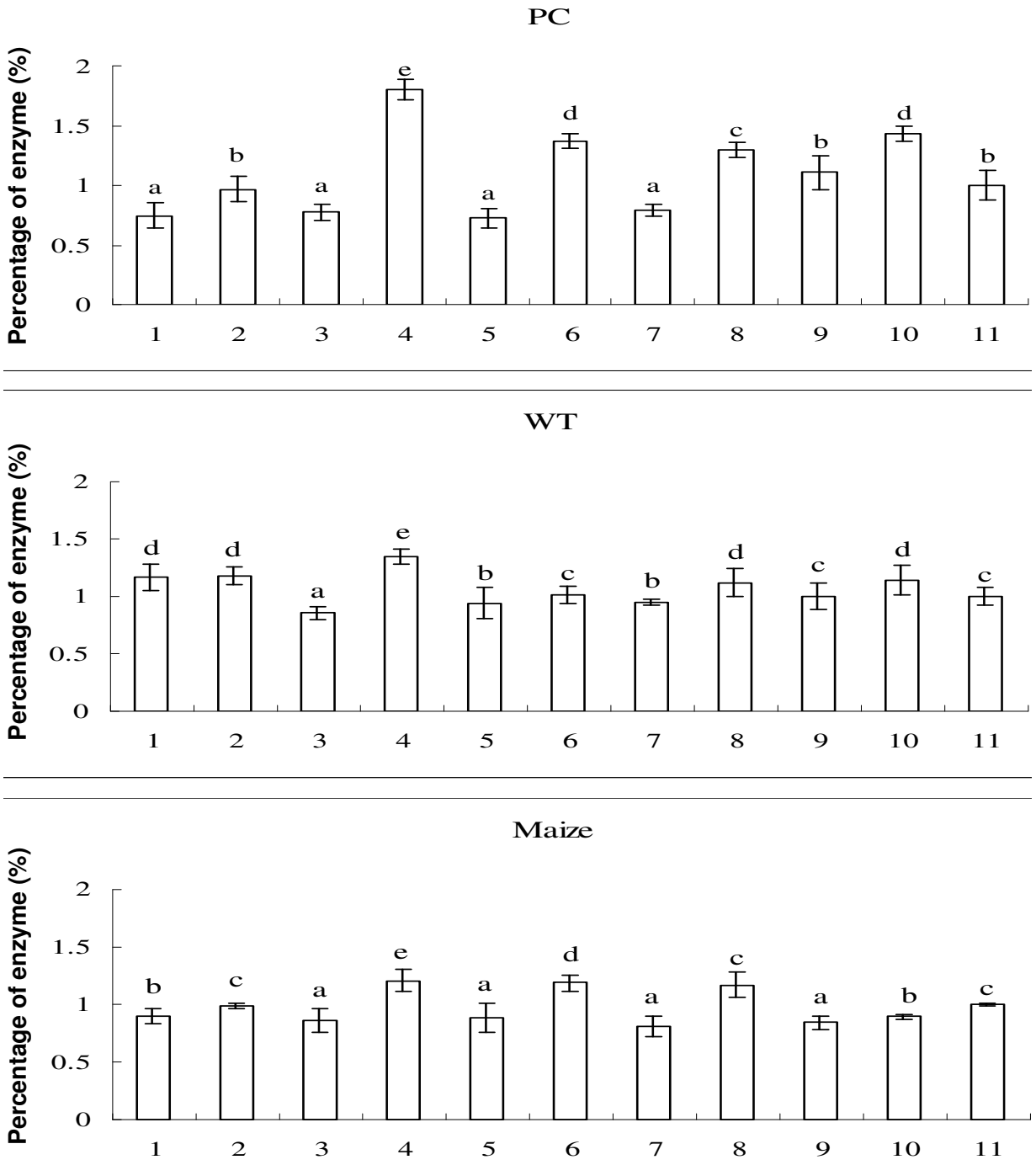

Figure 6. Effects of 1-butanol, neomycin and calcium ions on the activity of PEPC of the PC, WT and maize. Values followed by a different letter are significantly different at $P < 0.05$. 1, The treatment of 1-butanol; 2, the treatment of neomycin; 3, the treatment of EGTA; 4, the treatment of calcium; 5, the treatment of 1-butanol after treatment of EGTA; 6, the treatment of 1-butanol after treatment of EGTA and then treatment with calcium; 7, the treatment of neomycin after treatment of EGTA; 8, the treatment of neomycin after treatment of EGTA and then treatment with calcium; 9, the treatment of neomycin and 1-butanol after treatment of EGTA; 10, the treatment of neomycin and 1-butanol after treatment of EGTA and then treatment with calcium; 11, control. WT, wild type; PC, *pepc* transgenic rice.

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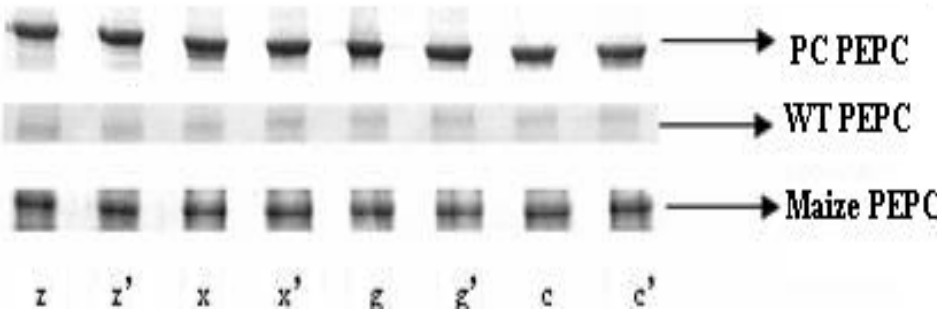

Figure 7. Effects of 1-butanol, neomycin and calcium on the PEPC protein expression of PC, WT and maize. WT, wild type; PC, *pepc* transgenic rice.

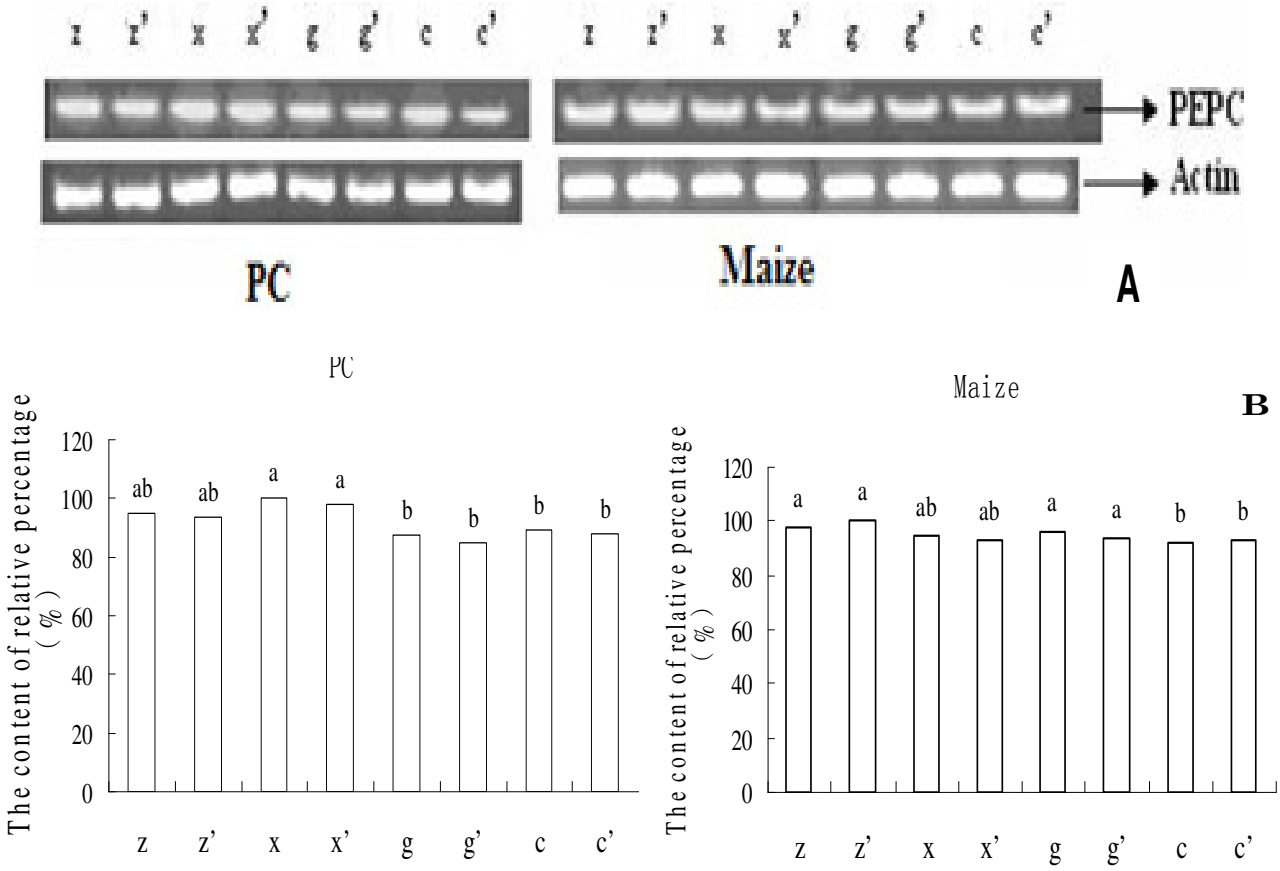

Figure 8. Effects of 1-butanol, neomycin and calcium on the *pepc* gene transcription of PC and maize. Values followed by a different letter are significantly different at the 0.05 probability level. WT, wild type; PC, *pepc* transgenic rice. Treatments are described as in Figure 1.

and attention.

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