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Enhanced drought tolerance in transgenic rice over-expressing of maize C_4 phosphoenolpyruvate carboxylase gene via NO and Ca^{2+}

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We determined the effects of endogenous nitric oxide and Ca^{2+} on photosynthesis and gene expression in transgenic rice plants (PC) over-expressing the maize C_4 *pepc* gene, which encodes phosphoenolpyruvate carboxylase (PEPC) under drought. In this study, seedlings were subjected to PEG 6000 treatments using PC and wild type (WT; Kitaake). The results showed that, compared with WT, PC had higher relative water content (RWC) and net photosynthetic rate (Pn) under drought. During a 2-day re-watering treatment, Pn recovered faster in PC than in WT. Further analyses showed that, under the drought treatment, the amount of endogenous hydrogen peroxide (H_2O_2) increased in WT mainly via NADPH oxidase. While in PC, the endogenous nitric oxide (NO) content increased via nitrate reductase and nitric oxide synthase on day 2 of the drought treatment and day 1 of the re-watering treatment. After 2 days of drought treatment, PC also showed higher PEPC activity, calcium content, phospholipase D (PLD) activity, *C_4-pepc* and *NAC6* transcript levels, and protein kinase activity as compared with PC without treatment. These changes did not occur in WT. Correlation analysis also proved NO associated with these indicators in PC. Based on these results, there was a particular molecular mechanism of drought tolerance in PC. The mechanism is related to the signaling processes via NO and Ca^{2+} involving the protein kinase and the transcription factor, resulted in up-regulation of PEPC activity and its gene expression, such as *C_4 pepc*. Some genes encode antioxidant system, *cu/zn-sod* as well, which promote antioxidant system to clear MDA and superoxide anion radical, thereby conferring drought tolerance.

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Introduction

Drought is one of the most important environmental stress factors limiting plant growth and crop yield (Terzi and Kadioglu, 2006). According to the available data, approximately 45% of the world's agricultural lands are subjected to frequent drought stress, and 38% of the world's human population resides in these areas (Ashraf and Foolad, 2007). Plants subjected to water stress can be more sensitive than unstressed plants to other biotic and abiotic stresses such as bacterial or fungal pathogens and competition from weeds. Thus, their productivity may be further limited under field conditions (Caruso et al., 2008). Hence, how to address problems associated with drought is an important focus of current research (Wardle, 2013).

The photosynthetic responses to drought involve interactions between physical and metabolic mechanisms (Pinheiro and Chaves, 2011). Compared with C_3 plants, C_4 plants have higher photosynthetic capacity, and higher nitrogen and water use efficiencies under drought conditions (Zhu et al., 2010). Therefore, researchers hope to introduce C_4 features into C_3 plants to improve their photosynthetic efficiency and yield (von Caemmerer et al., 2012).

Phosphoenolpyruvate carboxylase (PEPC) is a ubiquitous cytosolic enzyme in higher plants, and it is also widely distributed

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; DTT, dithiothreitol; DW, dry weight; EDTA, ethylene diamine tetraacetic acid; FW, fresh weight; Gs, stomatal conductance; H_2O_2, hydrogen peroxide; Ci, intercellular CO_2 concentration; MDA, malondialdehyde; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NR, nitrate reductase; $O_2^{-\bullet}$, superoxide anion radicals; •OH, hydroxyl radicals; PA, phosphatidic acid; PEPC, phosphoenolpyruvate carboxylase; PEPC-k, phosphoenolpyruvate carboxylase-kinase; PLD, phospholipase D; PPFD, photosynthetic photon flux density; Pn, net photosynthetic rate; POD, peroxidase; PVP, polyvinyl pyrrolidone; ROS, reactive oxygen species; Rubisco, ribulose-1,5-bisphosphate-carboxylase/oxygenase; RWC, relative water content; SOD, superoxide dismutase; TW, turgid weight; VPD, vapor pressure difference.

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in green algae and bacteria (Sage et al., 2012). In higher plants, there are several isoforms of PEPC with different organ specificities. These enzymes are involved in a variety of functions including stomata opening, fruit ripening, and seed maturation (Chollet et al., 1996). The leaves of C_4 and Crassulacean acid metabolism plants contain high levels of PEPC, which catalyzes the initial CO_2 fixation during photosynthesis (Fukayama et al., 2003). Previously, transgenic rice lines expressing high levels of the maize *C$_4$-pepc* gene (designated as PC lines) were successfully produced through transgenic technology (Ku et al., 1999). To date, several C_3 species have been genetically modified to overproduce PEPC (Miyao et al., 2011). Several studies have reported the detrimental effect of increased PEPC activity on Pn or biomass: for example, overproduction of *C$_4$-pepc* in rice resulted in reduced photosynthetic rates because of increased respiration under light conditions (Fukayama et al., 2003) and severely stunted growth (Chen et al., 2004). Other studies showed that PEPC-expressing transgenic plants have a relatively high Pn under stress conditions such as photo-oxidation, heat, and drought (Jiao et al., 2002, 2005; Bandyopadhyay et al., 2007; Fang et al., 2008; O'leary et al., 2011; Ling et al., 2014). Previous studies on PC lines showed that hydrogen peroxide (H_2O_2), calcium ions (Ca^{2+}), and nitric oxide (NO) donors play important roles in regulating stomata movement (Li et al., 2011; Ren et al., 2014; Chen et al., 2014). There are conflicting reports about the effects of PEPC overproduction on photosynthesis, with no clear consensus until now (Miyao et al., 2011). The different results of the various studies could be because of differences in the experimental or measurement procedures used, or because of differences in the expression level of the introduced *Zmpepc* among rice lines. Positional effects may have resulted in different expression levels of the trans-gene, in spite of the use of the constitutive *ZmUbi-1* promoter. Although there is some disagreement about the effects of the *C$_4$-pepc* trans-gene on photosynthesis, there is a general consensus that *C$_4$-pepc* transgene expression positively affects stress tolerance, although little is known about the underlying molecular mechanisms. In this study, analyses of transgenic rice overexpressing maize *C$_4$-pepc* (PC) and Kitaake (WT) provided evidence for the molecular mechanism of drought tolerance in PC. In PC, up-regulation of NO and down-regulation Ca^{2+} induces higher protein kinase activity for up-regulation of PEPC activity, enhances upstream gene expression of the transcription factor, such as *NAC6*, then induces higher downstream gene expression *C$_4$-pepc*, and *cu/zn-sod*, which encode components of the antioxidant defense system. The increased expression of these genes enhances the photosynthetic capability of the transgenic line under stress conditions.

Materials and methods

Plant materials and treatments

We used the transgenic rice (*Oryza sativa* L.) line PC, which over-expresses maize *pepc*. We used tenth generation plants (Jiao et al., 2002), which were derived from third generation plants (Ku et al., 1999). Wild-type (WT) rice plants (cv. Kitaake) were used as the control. The seeds were surface sterilized with 0.1% (w/v) mercuric chloride solution for 15 min, rinsed three times with 75% (v/v) ethyl alcohol, and then rinsed five times with distilled water. The sterilized seeds were germinated in Petri dishes on two layers of wet filter paper in a controlled incubator at 30 °C in darkness. After 4 days, seedlings were transferred to vessels containing 1/4 modified Hoagland solution with the following nutrients: KNO_3, 0.5 mM; $Ca(NO_3)_2$, 1.0 mM; KH_2PO_4, 1.0 mM; $MgSO_4$, 0.3 mM; H_3BO_3, 13.3 mM; $MnCl_2$, 3.0 mM; $CuSO_4$, 0.5 mM; $ZnSO_4$, 1.0 mM; Na_2MoO_4, 0.1 mM; NaCl, 2 mM; $CoCl_2$, 0.01 mM; $NiSO_4$, 0.1 mM; and Ethylenediamine-N,N_9-bis(2-hydroxyphenylacetic acid) Ferric sodium complex, 20 mM (Jones, 1982). The solution pH was adjusted to 5.8 (control condition) daily, and the solution was replaced every 2 day. When the seedlings reached the three-leaf stage, they were transferred to vessels containing full-strength modified Hoagland solution (Jones, 1982). The vessels were placed in a controlled growth chamber (14 h light/10 h dark photoperiod; light intensity, 600 μmol m^{-2} s^{-1}; 30 °C). When the sixth leaves from the base were fully expanded, they were collected and used for investigations.

Plants were subjected to a PEG-6000 treatment to simulate drought stress. The plants were transferred into culture solution containing 15% (w/v) polyethylene glycol (PEG)-6000. Then, after 2 days, the treated materials were transferred to a culture solution without PEG-6000 and were grown for a further 2 days. We evaluated the various parameters every day during the 4-day treatment. Each treatment was replicated three times. The sixth mature leaves from the base were collected at selected time points. The relative water content (RWC) of the fresh leaves was determined immediately. For other assays, leaf samples were frozen in liquid nitrogen (N_2) and stored at −75 °C until analysis.

Measurement of relative water content (RWC)

To measure RWC, 10 leaf discs (0.5 cm-diameter) per replicate were obtained from the central third of leaves using a circular cutter. The discs were weighed to obtain fresh weight (FW), and then floated on water at 4 °C in the dark. After 18 h, the turgid weight (TW) was measured. Leaf discs were then dried at 75 °C to constant weight and dry weight (DW) was obtained. The RWC (%) was calculated as follows:

$$\text{RWC} = (\text{FW} - \text{DW})/(\text{TW} - \text{DW}) \times 100(\text{Anjumet al.}, 2011).$$

Measurement of net photosynthetic rate (Pn)

Gas exchange was measured with an open gas-exchange system (LI-6400, Li-Cor, Lincoln, NE, USA). Illumination was provided by light-emitting diodes (470 and 665 nm; Li-Cor). The leaf-to-air vapor pressure difference (VPD) was controlled using a dew point generator (LI-610; Li-Cor). Measurements of net photosynthetic rate (Pn), stomata conductance (Gs), and intercellular CO_2 concentration (Ci) were performed under the following conditions: leaf temperature, 30 °C; 360 μmol mol^{-1} CO_2; 21% O_2; photosynthetic photon flux density (PPFD) 800 μmol m^{-2} s^{-1}; flow flux, 500 μmol s^{-1}; VPD, 1.0–1.2 kPa. Before all measurements, the uppermost fully expanded leaf was placed in the leaf chamber and exposed to 500 μmol m^{-2} s^{-1} PPFD at a leaf temperature of 30 °C in ambient air for 30 min. Each treatment was replicated three to five times (Li et al., 2011).

Antioxidant enzyme assays

Plant tissue (0.15 g, FW) was homogenized in 1.5 ml extraction buffer (50 mmol L^{-1} phosphate buffer, 1 mmol L^{-1} ethylene diamine tetraacetic acid (EDTA), 1% (w/v) polyvinyl pyrrolidone (PVP), pH 7.4) using a mortar and pestle on ice. The homogenate was centrifuged at 12,000 × *g* for 10 min at 4 °C, and the supernatant was used for assays. Superoxide dismutase (SOD) activity was assayed by measuring the ability of the enzyme in the crude extract to inhibit the photochemical reduction of nitroblue tetrazolium (NTB) by photo-chemically generated superoxide radicals. This reaction was monitored by measuring the change in absorbance at 560 nm using a UV-1200 spectrophotometer (Meipuda, Shanghai, China). One unit (U) of SOD was defined as the amount of enzyme required to inhibit the rate of nitroblue tetrazolium reduction by 50% at 25 °C (Giannopolitis and Ries, 1977). Peroxidase (POD) activity was

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