Journal of Plant Physiology 218 (2017) 206–221

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Improved short-term drought response of transgenic rice over-expressing maize C_4 phosphoenolpyruvate carboxylase via calcium signal cascade

Xiaolong Liu[a,b], Xia Li[a,b,*], Chuanchao Dai[c], Jiayu Zhou[c], Ting Yan[a,b], Jinfei Zhang[a]

[a] Institute of Food Crops, Provincial Key Laboratory of Agrobiology, Jiangsu Academy of Agricultural Sciences, Jiangsu High Quality Rice R and D Center, Nanjing Branch, China National Center for Rice Improvement, Nanjing 210014, PR China

[b] College of Life Science, Nanjing Agricultural University, Nanjing 210095, PR China

[c] Jiangsu Key Laboratory for Microbes and Functional Genomics, Jiangsu Engineering and Technology Research Center for Industrialization of Microbial Resources, College of Life Sciences, Nanjing Normal University, Nanjing 210023, PR China

ARTICLE INFO

Keywords:
Rice (*Oryza sativa* L.)
Drought
Phosphoenolpyruvate carboxylase
Signal transduction
Endogenous Ca^{2+}

ABSTRACT

To understand the link between long-term drought tolerance and short-term drought responses in plants, transgenic rice (*Oryza sativa* L.) plants over-expressing the maize *C_4-pepc* gene encoding phosphoenolpyruvate carboxylase (PC) and wild-type (WT) rice plants were subjected to PEG 6000 treatments to simulate drought stress. Compared with WT, PC had the higher survival rate and net photosynthetic rate after 16 days of drought treatment, and had higher relative water content in leaves after 2 h of drought treatment as well, conferring drought tolerance. WT accumulated higher amounts of malondialdehyde, superoxide radicals, and H_2O_2 than PC under the 2-h PEG 6000 treatment, indicating greater damages in WT. Results from pretreatments with a Ca^{2+} chelator and/or antagonist showed that the regulation of the early drought response in PC was Ca^{2+}-dependent. The NO and H_2O_2 levels in PC lines were also up-regulated via Ca^{2+} signals, indicating that Ca^{2+} in PC lines also reacted upstream of NO and H_2O_2. 2-h drought treatment increased the transcripts of *CPK9* and *CPK4* in PC via positive up-regulation of Ca^{2+}. The transcripts of *NAC6* [NACs (NAM, ATAF1, ATAF2, and CUC2)] and *bZIP60* (basic leucine zipper, bZIP) were up-regulated, but those of *DREB2 B* (dehydration-responsive element-binding protein, DREB) were down-regulated, both via Ca^{2+} signals in PC. PEPC activity, expressions of *C_4-pepc*, and the antioxidant enzyme activities in PC lines were up-regulated via Ca^{2+}. These results indicated that Ca^{2+} signals in PC lines can up-regulate the *NAC6* and *bZIP60* and the downstream targets for early drought responses, conferring drought tolerance for the long term.

1. Introduction

Drought is one of the major factors limiting the yield and productivity of crops (Hussain et al., 2011). There is genetic variability for the capacity to maintain yield and productivity under abiotic stress conditions in crop plants such as cereals (Dolferus, 2014). Plants respond to drought at physiological and molecular levels, which are apparent from analyses of their metabolomes and transcriptomes (Claeys and Inzé, 2013). Due to the complexity of drought stress responses regulated by multi-genes, progress in improving abiotic stress tolerance of crop plants using classic breeding and selection approaches has been slow. (Cattivelli et al., 2008; Mittler and Blumwald, 2010; Varshney et al., 2011; Dolferus, 2014; Wang et al., 2016).

Some C_4 plants, such as maize, show higher photosynthetic capacity and water-use efficiency under drought stress than do C_3 plants, such as rice (Zhu et al., 2010a). Because C_4 plants have a variety of mechanisms to cope with drought, researchers hope to introduce key C_4 genes into rice via genetic engineering to improve its photosynthetic efficiency,

Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; bZIPs, one of the basic leucine zippers; BSA, bovine serum albumin; bZIP60, basic leucine zipper 60; CaM, calmodulin; CAT, catalase; CAS, calcium-sensing receptor; CDPKs, Ca^{2+}-dependent protein kinase; chl, chlorophyll; C_i, intercellular CO_2 concentration; DREB, dehydration-responsive element-binding proteins; DW, dry weight; EGTA, glycol-bis(b-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; Gs, stomata conductance; GR, Glutathione reductase; NADP-MDH, NADP-dependent malate dehydrogenase; NADP-ME, NADP-dependent malic enzyme; NACs, NAM ATAF1 ATAF2 and CUC2; NBT, nitroblue tetrazolium; MAPK, mitogen-activated protein kinase; MDA, malondialdehyde; OAA, oxaloacetic acid; PEPC, phosphoenolpyruvate carboxylase; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; PLD, phospholipase D; PLC, phospholipase C; Pn, net photosynthetic rate; PPDK, pyruvate orthophosphate (Pi) dikinase; PPFD, photosynthetic photon flux density; PVP, polyvinyl pyrrolidone; qRT-PCR, quantificational real-time polymerase chain reaction; RLKs, receptor-like kinases; RWC, relative water content; RR, ruthenium red; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; TW, turgid weight; VPD, vapor pressure difference

* Corresponding author at: Institute of Food Crops, Provincial Key Laboratory of Agrobiology, Jiangsu Academy of Agricultural Sciences, Jiangsu High Quality Rice R and D Center, Nanjing Branch, China National Center for Rice Improvement, Nanjing 210014, PR China.

E-mail address: jspplx@jaas.ac.cn (X. Li).

http://dx.doi.org/10.1016/j.jplph.2017.08.005
Received 8 March 2017; Received in revised form 22 August 2017; Accepted 22 August 2017
Available online 31 August 2017
0176-1617/

drought tolerance, and yield (Zhu et al., 2010b; Karki et al., 2013). A set of genes encoding the key C_4 photosynthesis enzymes (phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31), pyruvate orthophosphate (Pi) dikinase (PPDK, EC 2.7.9.1), NADP-dependent malate dehydrogenase (NADP-MDH, EC 1.1.1.82), and NADP-dependent malic enzyme (NADP-ME, EC 1.1.1.40)) and genes regulating leaf anatomy have been introduced into, and expressed, in C_3 plants (He et al., 2005; Bandyopadhyay et al., 2007; Taniguchi et al., 2008; Thomas et al., 2012; Zhang et al., 2014; Doubnerová Hýsková et al., 2014). Most transgenic plants mentioned above were expressed at high levels and the enzymes remained functional (Jiao et al., 2003; Fukayama et al., 2003; Bandyopadhyay et al., 2007; Zhang et al., 2009; Ding et al., 2013; Karki et al., 2013; Ren et al., 2014; Zhang et al., 2014). Notably, most transgenic plants expressing these *C_4-pepc* genes at high levels were shown to be drought-tolerant, indicating that *C_4-pepc* is also a target for improving drought tolerance in rice (Zhang et al., 2009; Ding et al., 2013; Ren et al., 2014; Zhang et al., 2014, Liu et al., 2017). Our previous studies on transgenic rice plants expressing maize *C_4-pepc* at high levels showed that phosphatidic acid (PA), H_2O_2, NO-signaling molecules, and exogenous ATP participate in up-regulating PEPC activity and *C_4-pepc* expression, which will be helpful to regulate stomatal movement of PC for stress tolerance (Li et al., 2011; Chen et al., 2014; Ren et al., 2014; Huo et al., 2017). The improved drought tolerance of *C_4-pepc* was related to enhanced activities of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and ascorbate peroxidase (APX, EC 1.11.1.11) during a 2-d PEG-6000 treatment via Ca^{2+} (Qian et al., 2015a). These studies were based on PEG 6000 treatments lasting for days or months to simulate drought. However, changes in the levels of signaling molecules are early, and fast responses to drought that take place within a few seconds, minutes, or several hours, and rapid stress perception, can have important effects on reducing the damage of stress in plants. However, the early response of PC plants to drought stress is still unclear.

Ca^{2+} acts as a signal sensor stimulated by drought stress that triggers the downstream defensive signaling events mediated by phosphorylation cascades (Harper et al., 2004). Some biochemical approaches showed functions of calcium-dependent signals in *C_4-pepc* transgenic rice plants for days under drought conditions (Qian et al., 2015a, 2015b; Liu et al., 2017), but molecular evidence linking defined *C_4-pepc* with Ca^{2+}-regulated drought response at the whole-plant level in the short term (hours) has been lacking. The purpose of this work was to study the involvement of Ca^{2+} in the early stage responding to drought in PC rice plants, which will be helpful to establish a bridge between short-term (hours) and long-term (days) drought stress. We subjected PC or WT plants to simulated drought stress for days [15% (w/v) PEG 6000 for 16 d] or hours [5% PEG 6000 (w/v) for 120 min] in order to compare the similarities and differences between long-term (day) and short-term (hours) stress. The antioxidant enzyme activities, PEPC activity, and the transcript levels of some transcription factors such as *NAC6* [NACs (NAM, ATAF1, ATAF2 and CUC2)], *bZIP60* (basic leucine zipper, bZIP) and *DREB2 B* (dehydration-responsive element-binding protein, DREB), as well as some Ca^{2+} signal component including both protein kinase genes (*CPK9* and *CPK4*, genes of Ca^{2+}-dependent protein kinase, CDPK) and calmodulin (CAM) were analyzed within 2 h of PEG 6000 treatment. In order to determine the role of the extracellular calcium or calcium ions in the cell in the early stage of the drought response, we further pre-treated with glycol-bis(b-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA; a Ca^{2+} chelator) or ruthenium red (RR; an antagonist of sites of Ca^{2+} release) or both (i.e., EGTA + RR) combined with PEG treatment. Our results indicated that the signal cascade activated by Ca^{2+} in PC was involved in early drought responses, promoting its antioxidant system to clear superoxide anion radicals ($O_2^{\cdot -}$), conferring drought tolerance. This overarching continuum of models spanning from the signal to the cellular metabolism will be essential for successfully redesigning photosynthesis by *C_4-pepc* for drought stress tolerance in the future.

2. Materials and methods

2.1. Plant materials and culture conditions

We used the transgenic rice (*Oryza sativa var. Japonica* L.) line PC, which over-expresses maize *C_4-pepc*. We used 10th-generation plants (Ren et al., 2014), which were derived from third-generation plants described in detail elsewhere (Ku et al., 1999). Wild-type (WT) rice (*O. sativa var. Japonica* L.) plants (cv. Kitaake) were used as the control. Seeds of PC and WT were disinfested in 0.1% $HgCl_2$ for 15 min, washed three times with 75% ethanol, and disinfected in 75% ethanol for a further 5 min. Then, they were washed 3–5 times with distilled water and kept at 30 °C for 48 h to germinate. All seedlings were grown in International Rice Research Institute nutrient solution (Yoshida et al., 1976) in a growth room at 28 °C under an 8-h dark/16-h light photoperiod with light supplied at 200 μmol m^{-2} s^{-1}, and 50% relative humidity.

2.2. Long-term drought treatment and survival rate measurement

WT and PC seedlings with four leaves of uniform size were grown in a nutrient solution containing 15% PEG 6000 (based on preliminary tests using 10%, 15%, 20%, and 25% PEG) for 16 d. The number of surviving plants in each pot was counted at two days after rewatering. Survival rates were determined as described previously (Liu and Lin, 2013).

2.3. Reagents and treatment

All chemicals were purchased from Sigma–Aldrich (St Louis, MO, USA), and kits were used following the manufacturer's instructions, unless otherwise specified. Healthy rice seedlings with 4–5 leaves were prepared for the following experiments.

To understand the early and rapid responses of two rice lines to drought, we used a stem application method to introduce various reagents for two hours before a drought treatment. The treatment solutions, containing 10 mmol L^{-1} EGTA, 80 μmol L^{-1} RR, or both (i.e., EGTA + RR), were absorbed by the leaf sheath. The sheath of the seedlings was cut at the bottom and immediately placed in the solution, and then cut again at the base to remove air bubbles in the vascular bundles (Park et al., 1995). The detached seedlings without roots were kept in the solutions for 2 h and then exposed to 5% PEG 6000 for another 2 h. This concentration was selected based on preliminary experiments using 3%, 5%, 8%, and 10% PEG 6000 by measuring the relative water content (RWC) as the drought index. The 2-h drought treatments were conducted at 28 °C under a photosynthetic photon flux density (PPFD) of 200 μmol m^{-2} s^{-1}. Distilled water was used as the control (CK). Rice seedlings were harvested at 0, 10, 30, 60, 90, or 120 min after the drought treatment. Each treatment was repeated five times, with 25 seedlings per replication. After the treatments, the second leaves of the plants were immediately frozen in liquid N_2 and then stored at −80 °C until measurement. Three different biological experiments were conducted.

2.4. Measurements of net photosynthetic rate, stomatal conductance, and intercellular CO_2 concentration

Gas exchange was measured with an open gas-exchange system (LI-6400; Li-Cor, Lincoln, NE, USA). The leaf-to-air vapor pressure difference (VPD) was controlled using a dewpoint generator (LI-610; Li-Cor). The Pn (net photosynthetic rate), stomatal conductance (Gs), and intercellular CO_2 concentration (Ci) were measured under the following conditions: leaf temperature, 30 °C; 360 μmol mol^{-1} CO_2; 21% O_2; PPFD 800 μmol m^{-2} s^{-1}; flow flux, 500 μmol s^{-1}; and VPD, 1.0–1.2 kPa. Before measurements, the uppermost fully expanded leaf was placed in the leaf chamber and exposed to 500 μmol m^{-2} s^{-1} PPFD