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Full Length Article

Enhanced Tolerance of Transgenic Rice Plants Over-Expressing Maize C_4 Phosphoenolpyruvate Carboxylase Gene to Low Nitrogen Conditions

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Abstract

The present study identified and compared transgenic rice overexpressing maize C_4 *pepc* (PC) with wild-type (WT; Kitaake) in low nitrogen tolerance through a combination of physiological, metabolic and proteomic analysis. The results showed that PC had smaller decreases and higher dry weight and net photosynthetic rate under low nitrogen conditions compared with WT. The increased photosynthetic rate in PC was associated with higher phosphoenolpyruvate carboxylase (PEPC) activity, *pepc* gene expression, and antioxidant system enhancement. The transgenic type with PC accumulated less starch or soluble sugars than WT under low nitrogen conditions. Metabolic analysis showed elevated PEPC activity in PC increased the synthesis of malate, citrate and 2-oxoglutarate under low nitrogen conditions. This increase also enhanced enzyme activities of the anaplerotic pathway for the tricarboxylic acid cycle and nitrogen assimilation. Furthermore, leaf N level of PC was higher than WT. Using 2-DE, 12 differentially expressed spots were identified between N deficient PC and WT. The proteins involved in photosynthesis, tricarboxylic acid cycle, amino acid biosynthesis, ROS scavenging, and translation expressed in higher concentration in N deficient PC. The increased carbon flow towards nitrogen metabolism could alleviate the inhibition of photosynthesis and had higher N content in PC under low nitrogen conditions, indicating enhanced tolerance of transgenic type PC to low nitrogen compared with WT.

Keywords: Phosphoenolpyruvate carboxylase; Transgenic rice; Low nitrogen tolerance; Photosynthesis; Carbon metabolism; Nitrogen metabolism

Introduction

In current agricultural practices, crop productivity relies heavily on nitrogen (N) fertilization. However, some environmental effects like water pollution and an imbalance of nutrients in ecosystems have been produced by using large amounts of N fertilizer (Miller and Cramer, 2005). It is essential for the development of sustainable agriculture to increase nitrogen use efficiency of plants (Xu *et al*., 2012; Chen *et al*., 2018). However, it is difficult to balance the evolutionary tradeoff between high productivity and adaptation to low nitrogen in most current cultivars such as rice selected for use in N-rich environments (Raun and Johnson, 1999). Improving N assimilation of crops is one of possible methods to solve these problems.

There have been some strategies to improve N assimilation using biotechnology in rice. N uptake could be increased by over-expressing of ammonium transporter *OsAMT1-1* in rice, was albeit with growth impairment (Hoque *et al*., 2006) and decrease in biomass (Kumar *et al*., 2006). Under low-nitrogen conditions, the *ZmDof1* (Maize, a plant-specific transcription factor) expression up-regulated the phosphoenolpyruvate carboxylase (PEPC) gene expression, enhance nitrogen assimilation, alter carbon and nitrogen metabolites in rice (Kurai *et al*., 2011). Selvaraj *et al.* (2017) reported that over-expression of the barley alanine aminotransferase gene (*HvAlaAT*) can improve nitrogen use efficiency (NUE) with no undesirable growth phenotypes in rice. In the transformants expressing *ZmDof1* or *HvAlaAT* above, the increase of N content was both observed as well as plant biomass and yield.

The responses to low nitrogen condition involve interactions between physiological and molecular mechanisms (Zhu *et al*., 2010). In comparison with C_3 plants, the higher photosynthetic capacity has been found in C_4 plants, as well as increased N and water use efficiencies (Caemmerer *et al*., 2012). Therefore, C_4 features may be introduced to improve photosynthetic efficiency, NUE, and yield of C_3 plants (Long *et al*., 2015).

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Phosphoenolpyruvate carboxylase (PEPC) located in cytosol, is distributed in green algae, bacteria and higher plants (Izui *et al*., 2011). In C_4 plants, PEPC is the primary enzyme used for fixing atmospheric CO_2 and highly regulated by metabolite levels (Caemmerer and Furbank, 2016). However, the primary function of PEPC is anaplerotic in C_3 plants, replenishing the tricarboxylic acid cycle (TCA) with intermediates withdrawn for use in N assimilation (Masumoto *et al*., 2010). The increase of the supply of 4-carbon carboxylic acids has been found in Arabidopsis overexpressing PEPC, which provided increased amino acid and protein synthesis with carbon skeletons. The overexpression also led to the increase of CO_2 assimilation rate, dry weight and starch content (Kandoi *et al*., 2016).

Transgenic rice lines have been successfully produced through transgenic technology, expressing high levels of the maize C_4-*pepc* gene (Ku *et al*., 1999). It has been reported that *pepc* transgenic rice showed higher photosynthetic rates (Ku *et al*., 2000) and grain yield per plant when compared with untransformed one (Jiao *et al*., 2001). Moreover, C_4 *pepc*-overexpressing transgenic rice showed a relatively high photosynthesis and enhanced tolerance under photo-oxidation, heat and drought stresses (Jiao *et al*., 2005; Bandyopadhyay *et al*., 2007; Qian *et al*., 2015). The elevated PEPC activity also induced the activities of carbon (C) and N metabolism enzymes in transgenic rice under natural conditions (Li and Wang, 2013). Shi *et al.* (2015) reported that PEPC in leaves is very important in regulating the balance of C and N metabolism in C_3 plants. However, the low nitrogen tolerance has not been tested in C_4-*pepc* transgenic rice. Little information is available on low nitrogen tolerance of transgenic rice. In the present study, the transgenic rice overexpressing maize C_4-*pepc* and untransformed wild-type rice were used as experimental materials. The goal was to examine whether C_4-*pepc* transgenic rice was more tolerant to low nitrogen conditions than untransformed rice based on some physiological, metabolic and proteomic evidence. This study will be helpful for us to breed rice varieties with simultaneously high photosynthetic efficiency under low N condition.

Materials and Methods

Plant Materials and Treatments

One transgenic rice (*Oryza sativa L.*) line PC, which over-expresses maize *pepc* was used with WT rice (cv. Kitaake) as the control. The tenth-generation plants used were derived from their third generation (Ku *et al*., 1999). The sterilized seeds were germinated in plates of filter paper moistened with water and incubated at 30°C in darkness. Seedlings of WT and PC were then grown in ion-exchanged water after 4 days in a controlled growth chamber (MLR-351H, Sanyo, Osaka, Japan) (14 h light/10 h dark [14/10 h] photoperiod; 28°C in light, 22°C in dark). When the seedlings reached at three leaf stage, transferred to vessels containing Yoshida nutrient solution and used for further investigations.

Full-strength Yoshida nutrient solution ingredients were used for N-sufficient treatment (MN) in 3 m*M* N (Yoshida *et al*., 1976). Full strength was maintained with the exception of N concentration, which reduced to a final concentration of 0.7 m*M* N for N-deficient treatment (LN). The pH was adjusted to 5.8 daily and the solution was exchanged every 2 days. At 14 days after flowering, plants from WT and PC were sampled between 9:00 and 11:00 am time consistent with the gas exchange measurement. For dry weight, the samples included aerial parts except for the grains of rice plants. For other assays, flag leaves were stored at −75°C until analyzed.

Measurement of Dry Weight, Soluble Sugar and Leaf N Content

The dry weight was determined in rice shoots following the methods of Li *et al*. (2011). Soluble sugar and starch contents were measured in flag leaves following the method as for phenols using spectrophotometer (Cai and Yuan, 1982). The N levels were measured in leaves with a model FLASH 2000 NC analyzer (Thermo Fisher Scientific, Waltham, MA) by the method of Kurai *et al*. (2011).

Measurement of Gas Exchange and SPAD

Gas exchange was measured with LI-6400 photosynthesis system (Li-Cor, Lincoln, NE, U.S.A.). Net photosynthetic rate (Pn), 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; photosynthetic photon flux density (PPFD) 1000 μmol m^{-2} s^{-1}; flow flux, 500 μmol s^{-1}; vapor pressure difference (VPD), 1.0–1.2 kPa. Flag leaves were placed in the leaf chamber and exposed to 500 μmol m^{-2} s^{-1} PPFD at leaf temperature of 30°C in ambient air for 30 min before measurements. Each treatment was repeated three to five times (3–5 flag leaves). SPAD values were measured on flag leaves with a chlorophyll meter. Each measurement was conducted on five replicates in each treatment.

Determination of PEPC and Rubisco Activity

PEPC activity was measured by the method of Giglioli-Guivarc'h *et al*. (1996). The mixture was shaken before reading absorbance. The enzyme activity was calculated from the absorbance value at 340 nm. Rubisco activity was assayed as described by Wei *et al*. (1994) and Kung *et al*. (1980). The steps were all carried out at 4°C. The enzyme activity was measured by adding some amounts of $NaHCO_3$ and calculated from an absorbance value at 340 nm.

Extraction of Total RNA and Quantitative PCR

Total RNA was extracted using an RNA simple Total RNA

Kit (Tiangen, Beijing, China). A PrimeScript RT Master Mix Perfect Real Time Kit (TaKaRa, Dalian, China) was used to conduct reverse transcription reactions and qRT-PCR analyses were conducted using an SYBR Premix Ex TaqTM II kit (TaKaRa). Applied Bio-systems instruments (Applied Biosystems, Foster City, CA, USA) were used for these analyses. The sequences of gene primers are shown in Table 1.

Table 1: Genes and primers for RT-PCR

Gene	Primer
pepc	5'-AGCTCCACAGTTCGTCTGGT-3'(forward) 5'-GCTCAAGTGGCTCAAGGAAC-3'(reverse)
Actin	5'-CCCTCTTTCATCGGTATGGA-3'(forward) 5'-TTGATCTTCATGCTGCTTGG-3' (reverse)

Assessment of C Metabolism Enzymes

Carbonic anhydrase (CA) activity was measured as described by Guo *et al*. (1988). The activity was calculated from the change rate in pH of the reaction solution. NADP-malate dehydrogenase (NADP-MDH) activity was determined according to Li *et al*. (1987). The reaction was performed at 30°C and the oxidation of NADPH was monitored by absorbance at 340 nm. NADP-isocitrate dehydrogenase (NADP-ICDH) activity was measured by the method of Lu *et al*. (2005). One unit of activity was defined as the amount of enzyme which catalyzed the production of 1 μmol NADPH min^{-1} at 340 nm.

Determination of Metabolites Levels

Metabolites (malate, citrate and 2-oxoglutarate) analyses were carried out by the method of Roessner *et al*. (2001). The leaf powder was extracted with 100% methanol. An internal standard (ribitol solution) was added for quantification. The derivative was injected into a gas chromatography-mass spectrometry system (Agilent 5975C, Agilent Technologies, Santa Clara, CA, USA). The amounts of absolute metabolite were determined using calibration curves obtained with standard compound mixtures.

Assessment of N Metabolism Enzymes

The measurement of nitrate reductase (NR) activity was conducted by the method of Riens and Heldt (1992). One unit of activity was the amount of enzyme catalyzing the production of 1 μmol NO_2^- per min at 37°C. Glutamine synthetase (GS) activity was measured as described by Jin *et al*. (2007). One unit of GS activity was the amount of enzyme catalyzing the formation of 1 μmol γ-glutamyl hydroxamate per min at 37°C. Glutamate synthase (GOGAT) activity assay was performed as described by Jamai *et al*. (2009). One unit of GOGAT activity was the amount of enzyme catalyzing the oxidation of 1 μmol NADPH per min at 30°C.

Assays of Antioxidant Enzymes

Superoxide dismutase (SOD) activity was assayed by measuring the change in absorbance at 560 nm. One unit 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 measured by the method of Gao *et al*. (2008). One unit of POD was defined as the amount that caused a 0.1 OD increase in the absorbance value per min at 470 nm. Catalase (CAT) activity was determined by the method of Jiang and Zhang (2001). One unit of CAT was defined as the amount which caused a 0.1 decrease in absorbance per min at 240 nm. The soluble protein content was estimated as described by Bradford (1976), using bovine serum albumin as the standard.

Determination of H_2O_2 and Malondialdehyde (MDA) Contents

The H_2O_2 content was measured by the method of Patterson *et al*. (1984) and Ren *et al*. (2014). The solution was measured at 410 nm, and H_2O_2 content was calculated from a standard curve using known concentrations of H_2O_2. MDA was determined as an indicator of lipid peroxidation. The absorbance of the reaction mixture was measured at 532 nm by the method of Hodges *et al*. (1999).

Protein Extraction and 2-DE Analysis

Leaf proteins were extracted using a TCA/acetone procedure. The protein concentration was determined using Bio-Rad protein assay reagent with BSA as the standard. 100 μg of total protein was loaded onto each IPG strip. The first dimension isoelectric focusing (IEF) was performed on IPG strips (GE Healthcare, U.S.A.) with pIs from 3 to 10. The IPG strips were reduced and alkylated after their focus and the proteins were separated on SDS-PAGE gels (12.5% acrylamide, GE Healthcare, U.S.A.) without a stacking gel. Triplicate gels were run for each treatment and stained with CBB (Coomassie Brilliant Blue) G-250. An ImageScanner (GE Healthcare, U.S.A.) was used to capture gel images. Scanned gel images were imported into ImageMaster 2D (GE Healthcare, U.S.A.), warped and matched. The matched spots were quantified based on the average normalized volumes of three gels. A criterion of $P < 0.05$ and an abundance ratio of at least 1.2 was used to define significant differences between species or treatments. The protein spots with the ratio of at least 1.5 were selected for further identification.

MS Analysis and Database Searching

Spots were manually excised from gels and destained.

The proteins were reduced with DTT, alkylated with iodoacetamide and digested with trypsin. Mass spectra were acquired on an Ultraflex II MALDI-TOF/TOF tandem mass spectrometer (Bruker Daltonics, U.S.A.) and internally calibrated using tryptic peptides from autodigestion. A Mascot Server was used to submit mass spectrum data to online database searches for protein identification. Two protein databases were included: an updated compilation of UniProt and non-redundant (NCBInr) database. The proteins were searched first against *Oryza sativa* and then against green plants.

Statistical Analysis

The data were analyzed by one-way ANOVA using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The $2^{-\Delta\Delta Ct}$ method was used to analyze qRT-PCR data. The $P < 0.05$ was considered as significant differences between species or treatments.

Results

Plant Physiological Response to Low N

The change in the shoot dry weight at 14 days after flowering was similar for both plant types under MN (Fig. 1a). However, the decrease of dry weight in PC was less than in WT under LN suggesting an improved harvest index of PC. At LN conditions, the decrease in Pn of PC was less than WT (Fig. 2a) and PC showed the higher value of Pn than WT. The Gs and Ci increased in PC rice under LN compared with WT (Fig. 2b and c). While SPAD value of PC was similar to WT in leaves (Fig. 1b).

Proteins and Gene Expression in Transgenic Rice

The PC showed much higher PEPC activity than WT under LN and the transcriptional level of *C_4-pepc* increased in PC significantly (Fig. 3a and b). Rubisco activity also showed higher values in PC and more increase than in WT (Fig. 3c) under low N conditions.

Soluble Sugar, Starch and N Contents in Transgenic Rice under Low Nitrogen

The amounts of soluble sugar decreased in PC and the higher in WT than in PC (Fig. 4a). Starch content declined in PC and WT under LN, but showed similar levels of starch content in PC with WT under MN or LN (Fig. 4b). The nitrogen concentrations of leaves showed significant difference in N utilization between PC and WT under 0.7 m*M* of N and these values were higher in PC than in WT (Fig. 4c).

Organic Acids Accumulation

The malate contents showed no significant change in PC and WT under LN treatment (Fig. 5a). Compared to WT, PC

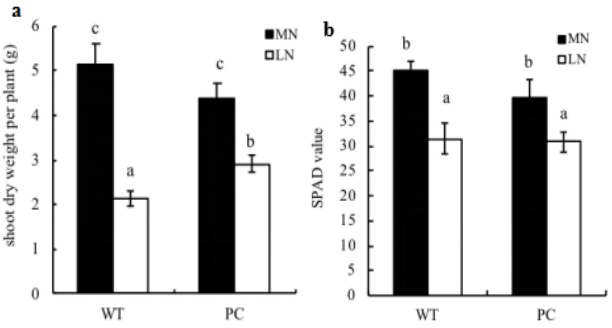

Fig. 1: Shoot dry weight per plant and SPAD value of WT and PC under low nitrogen conditions. (**a**) Changes in dry weight both in WT and PC under 0.7 m*M* of N at 14 days after flowering. (**b**) Changes in SPAD value both in WT and PC under the same conditions as (a)

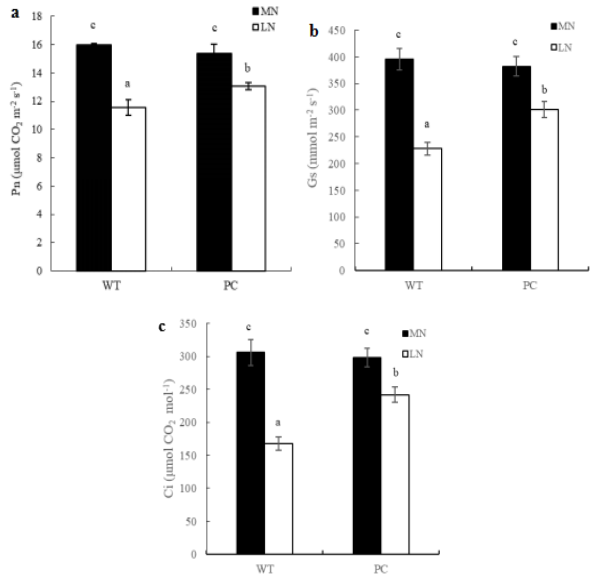

Fig. 2: Net photosynthetic rate (Pn), stomata conductance (Gs) and intercellular CO_2 concentration (Ci) of WT and PC under low nitrogen conditions. (**a**) Changes in net photosynthetic rate both in WT and PC under 0.7 m*M* of N at 14 days after flowering. (**b**) Changes in stomata conductance both in WT and PC under the same conditions as (a). (**c**) Changes in intercellular CO_2 concentration both in WT and PC under the same conditions as (a)

exhibited higher levels of malate under low nitrogen condition. This might indicate that oxalacetic acid (OAA) was decreased, because it could be converted into malate by malate dehydrogenase. There was more decrease in citrate levels of WT than PC under LN treatment (Fig. 5b). The 2-oxoglutarate (2-OG) content decreased slightly in WT, while a marked increase was observed in PC under low nitrogen conditions (Fig. 5c).

C and N Metabolic Enzymes Activities

The nitrate reductase (NR) activity of PC and WT did not

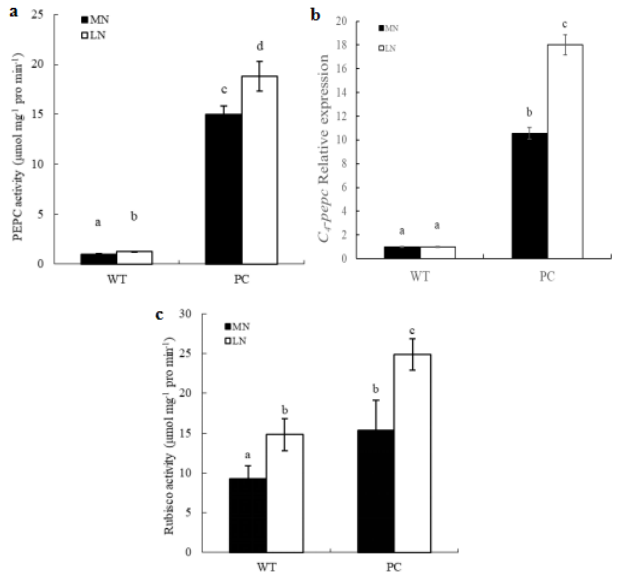

Fig. 3: PEPC activity, *C_4-pepc* gene expression and Rubisco activity of WT and PC under low nitrogen conditions. (**a**) PEPC activity both in WT and PC under 0.7 m*M* of N at 14 days after flowering. (**b**) *C_4-pepc* relative expression both in WT and PC under the same conditions as (a). (**c**) Rubisco activity both in WT and PC

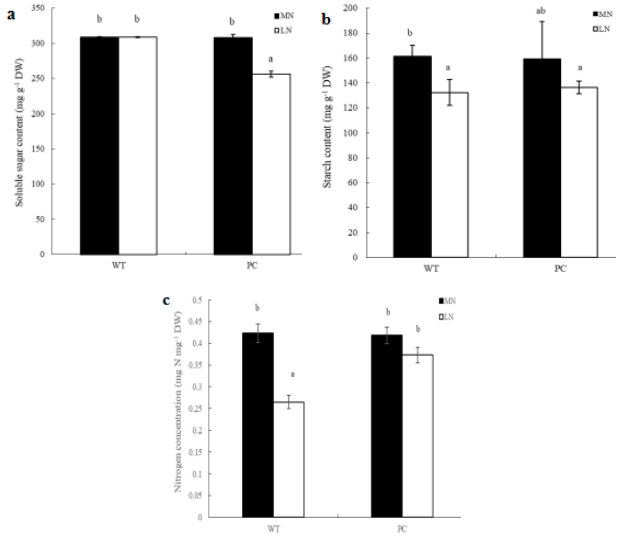

Fig. 4: Soluble sugar content, starch content and nitrogen concentration of WT and PC under low nitrogen conditions. (**a**) soluble sugar content both in WT and PC under 0.7 m*M* of N at 14 days after flowering. (**b**) starch content both in WT and PC under the same conditions as (a). (**c**) nitrogen concentration both in WT and PC under the same conditions as (a)

decrease significantly; however, higher levels were found in PC at LN treatment (Fig. 6a), showing an improvement of NR activity under LN. The GS activity decreased significantly in WT, while increased to greater level in PC at LN treatment (Fig. 6b). Meanwhile, the increase of GOGAT

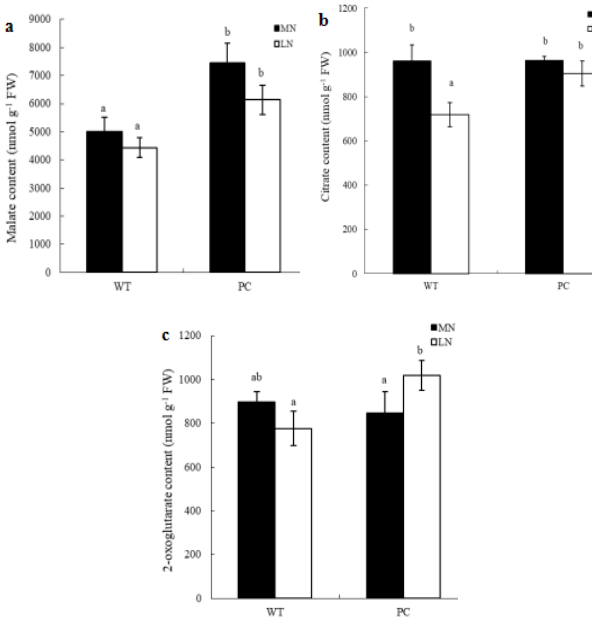

Fig. 5: Malate content, citrate content and 2-oxoglutarate content of WT and PC under low nitrogen conditions. (**a**) malate content both in WT and PC under 0.7 m*M* of N at 14 days after flowering. (**b**) citrate content both in WT and PC under the same conditions as (a). (**c**) 2-oxoglutarate content both in WT and PC under the same condition

activity was larger in PC than in WT and a higher level of GOGAT activity was observed in PC at LN conditions (Fig. 6c). The higher CA activity was observed in PC under both MN and LN and PC had more increase than WT (Fig. 7a). The activities of NADP-MDH and NADP-ICDH showed no obvious increases in both PC and WT (Fig. 7b and c). However, PC had higher activities of two enzymes than WT at LN conditions. These results showed that overexpression of PEPC could induce the activities of enzymes in C and N metabolism under LN.

Antioxidant Enzymes Activities

Lipid peroxidation as indicated by MDA content in plants was higher in PC than in WT under MN (Fig. 8a). Under LN conditions, the MDA content increased in WT, and showed a significant decrease in PC, reaching the same level as for WT. Under LN conditions, the H_2O_2 content significantly increased in WT, while a small decrease was observed in PC (Fig. 8b). The SOD (Fig. 9a), POD (Fig. 9b) and CAT (Fig. 9c) activities decreased after LN treatment in WT, while the changes were not significant in PC. PC had higher activities of antioxidant enzymes than WT under LN.

Identification of Proteins and 2-DE Analysis

Using the Image-Master software, there were about 2500 protein spots detected reproducibly. There were 32 up-

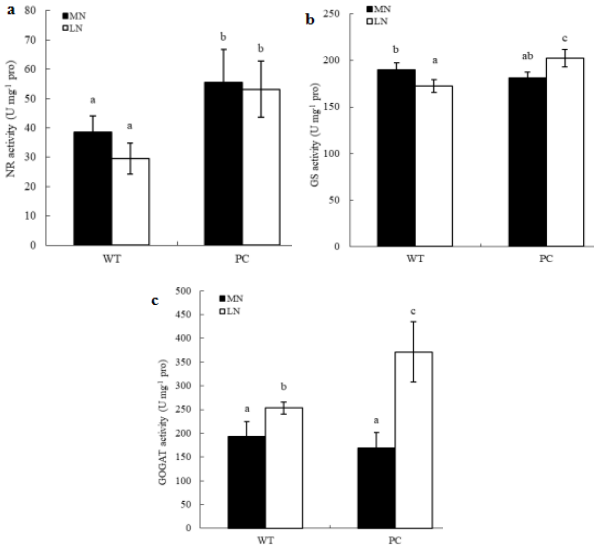

Fig. 6: NR activity, GS activity and GOGAT activity of WT and PC under low nitrogen conditions. (**a**) NR activity both in WT and PC under 0.7 m*M* of N at 14 days after flowering. (**b**) GS activity both in WT and PC under the same conditions as (a). (**c**) GOGAT activity both in WT and PC under the same conditions as (a)

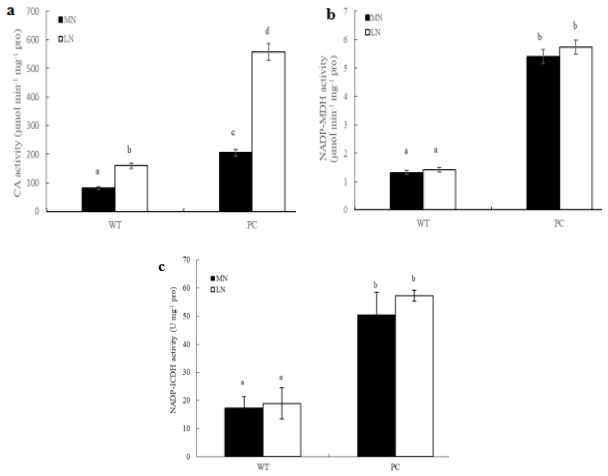

Fig. 7: CA activity, NADP-MDH activity and NADP-ICDH activity of WT and PC under low nitrogen conditions. (**a**) CA activity both in WT and PC under 0.7 m*M* of N at 14 days after flowering. (**b**) NADP-MDH activity both in WT and PC under the same conditions as (a). (**c**) NADP-ICDH activity both in WT and PC under the same conditions as (a)

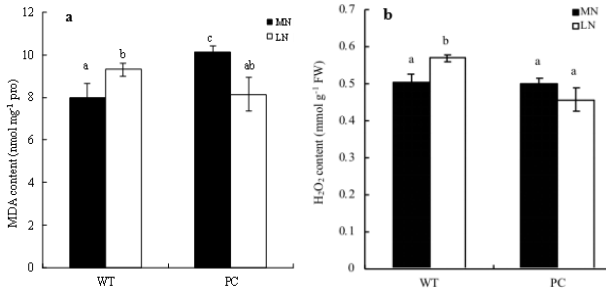

Fig. 8: MDA content and H_2O_2 content of WT and PC under low nitrogen conditions. (**a**) MDA content both in WT and PC under 0.7 m*M* of N at 14 days after flowering. (**b**) H_2O_2 content both in WT and PC under the same conditions as (a)

regulated spots and 13 down-regulated spots under LN when compared with PC and WT. The 12 differentially expressed protein spots were further identified by an MS analysis based on the criterion of $P < 0.05$ and at least 1.5 fold change. These spots are shown in representative gels of PC and WT (Fig. 10). There was no effective PMF data of one spot (D1), which made it not be identified and further analysis will be required. The 11 differentially expressed protein spots were identified, including 8 up-regulated spots and 3 down-regulated spots (Table 2).

Discussion

C_4-phosphoenolpyruvate carboxylase genes have been transferred from maize into C_3 rice successfully (Matsuoka *et al*., 2001). Increased photosynthetic capacity or enhanced stress tolerance was found in transgenic rice by overexpression of maize *pepc* gene under N-sufficient condition (Chen *et al*., 2014; Qian *et al*., 2015). Whether low nitrogen tolerance of PC would be better than WT remains unclear. In this study, 0.7 m*M* of N was induced as the LN concentration and 3 m*M* of N as the MN concentration to investigate the effects of low nitrogen on PC. There was found more inhibition in photosynthesis of WT and PC showed better resistance at LN conditions than WT.

Nitrogen can be partitioned to Rubisco (Makino, 2011) and chlorophyll significantly in rice (about 30%), which affect photosynthetic rate, so its deficiency could inhibit biomass production. In the present study, the decrease of Pn in WT was more than in PC under LN. Compared with WT, the increase in biomass under LN indicated an elevated Pn of PC because about 90% of biomass is produced by net photosynthesis (Zelitch, 1982). It was further investigated that increased levels of stomatal factors, not changes in chlorophyll, were attributed to higher Pn of PC at LN conditions.

The PEPC activities were enhanced by CO_2 in plants under abiotic and biotic stress (Doubnerova and Ryslava, 2011) and its affinity for C was higher than Rubisco (Pollastrini *et al*., 2014). Chi *et al*. (2001) reported that *C_4-pepc* transgenic rice possessed an increased activity of PEPC and an enhanced C_4 photosynthesis under N-sufficient conditions. The expression of *C_4-pepc* gene, the activities in PEPC and carbonic anhydrase of PC were induced, reached higher levels than WT and increased more than Rubisco activities at LN conditions. The results indicated that the enzymes of C_4 photosynthesis might be more tolerant to low nitrogen than those involved in C_3 photosynthesis and could account for elevated photosynthetic capability in PC.

Table 2: Identification of differentially expressed proteins in C_4 *pepc* transgenic rice under low nitrogen conditions by MS

Spot no.	Accession no [a].	Protein name	Score	Fold[b]	Function	Organism
U1	gi|1261858	catalase	504	2.20	Hydrogen peroxide catabolic process	*Oryza sativa*
U2	gi|1261858	catalase	629	1.55	Hydrogen peroxide catabolic process	*O. sativa*
U3	gi|344017	ribulose1,5-bisphosphatecarboxylaselarge subunit	332	1.75	Photosynthesis	*O. sativa*
U4	gi|357164174	isocitrate dehydrogenase [NAD] regulatory subunit 1, mitochondrial isoform X1	195	2.73	Tricarboxylic acid cycle	*Brachypodium distachyon*
U5	gi|357113972	phosphoserine aminotransferase 1, chloroplastic	263	2.42	Amino acid biosynthesis	*B. distachyon*
U6	gi|357149358	2-Cys peroxiredoxin BAS1, chloroplastic	85	1.75	Hydrogen peroxide catabolic process	*B. distachyon*
U7	gi|1162449977	enolase-phosphatase E1	75	1.62	Amino acid biosynthesis	*Zea mays*
U8	gi|357112267	40S ribosomal protein S21	217	1.60	Translation	*B. distachyon*
D2	gi|344017	ribulose1,5-bisphosphatecarboxylaselargesubunit	220	1.82	Photosynthesis	*O. sativa*
D3	gi|57283874	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	112	1.66	Photosynthesis	*O. sativa*
D4	gi|9230755	pathogenesis-related protein PR-10a	204	1.80	Defense response	*O. sativa Indica*

[a] Accession number in NCBI

[b] Fold changes in levels of proteins were calculated by comparing intensities of spots between PC and WT under low nitrogen

Plants under LN can also suffer from oxidative stress because of more production of reactive oxygen species (ROS). In this study, increased lipid peroxidation (MDA and H_2O_2) led to more oxidative stress in WT at LN conditions, while this stress was less in PC due to decreased levels of those products. Meanwhile, the activities of the antioxidant enzymes (SOD, POD and CAT) decreased in WT under LN, while showed no obvious difference in PC between MN and LN. The results suggested that PC was more resistant to oxidative stress under LN. This tolerance was consistent with other stress condition such as photoinhibition (Jiao *et al*., 2005) or drought (Gu *et al*., 2013), which might be attributed to increased Pn in PC compared with WT.

The PEPC is also crucial for C and N metabolism in plant leaves (Shi *et al*., 2015). In the leaves of *C_4-pepc* transgenic rice, the increased PEPC activity could induce some changes in the anaplerotic pathway of TCA cycle under low nitrogen condition. NADP-MDH possibly converts OAA synthesized by PEPC into malate, which can be used in the TCA cycle. NADP-ICDH is one of key enzymes of TCA. PC showed increased activities of NADP-MDH and NADP-ICDH when compared with WT at LN conditions. The results were in agreement with the changes in activities of these enzymes in the increased PEPC activity of transgenic rice introduced by *ZmDof1* gene (Kurai *et al*., 2011) and indicated that overexpression of *C_4-pepc* gene could regulate C flux to the TCA cycle of transgenic rice. Some related organic acids in the TCA cycle were also determined. Leaf metabolite analysis showed that the elevated PEPC activity increased the levels of malate and citrate under low nitrogen in PC when compared with WT. As an intermediate of C and N metabolism, the content of 2-OG influenced N uptake and assimilation (Yuan *et al*., 2007). There was more increase in 2-OG of PC under low nitrogen condition. These results indicated that the increased PEPC activity could significantly induce the anaplerotic flux to malate and hence 2-OG.

Previous studies demonstrated that C flow was redirected largely from soluble sugars and starch to organic

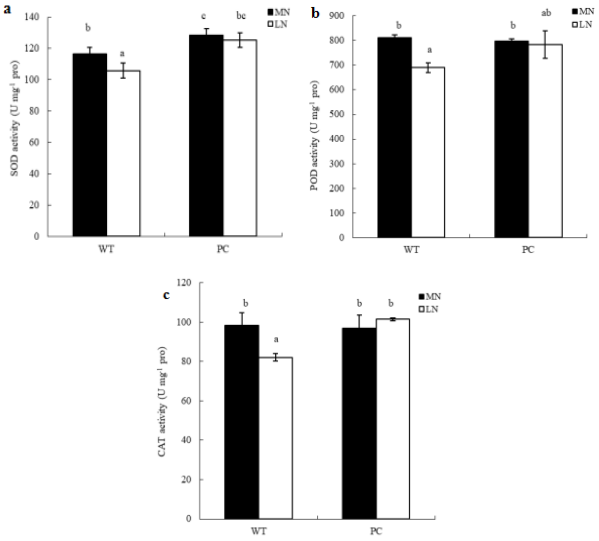

Fig. 9: SOD activity, POD activity and CAT activity of WT and PC under low nitrogen conditions. (**a**) SOD activity both in WT and PC under 0.7 m*M* of N at 14 days after flowering. (**b**) POD activity both in WT and PC under the same conditions as (a). (**c**) CAT activity both in WT and PC under the same conditions as

acids of transgenic plants overexpressing *pepc* gene under low nitrogen conditions (Miyao and Fukayama, 2003). Similar results were observed in PC under low nitrogen condition. The levels of soluble sugars in PC were lower than in WT, while PC and WT showed similar levels of starch. In addition, PC showed higher level of leaf N concentration than WT under LN, which suggested more N assimilation in PC. Meanwhile, the increased activities of NR, GS and GOGAT suggested that N fixation and ammonium assimilation were enhanced in PC under low nitrogen condition. Some forms of PEPC have been reported in metabolic engineering studies with feedback inhibition in the transgenic potato (Rademacher *et al*., 2002) and *Arabidopsis* (Chen *et al*., 2004).

Taken together, the metabolic changes increased C flux from starch and soluble sugars into organic acids

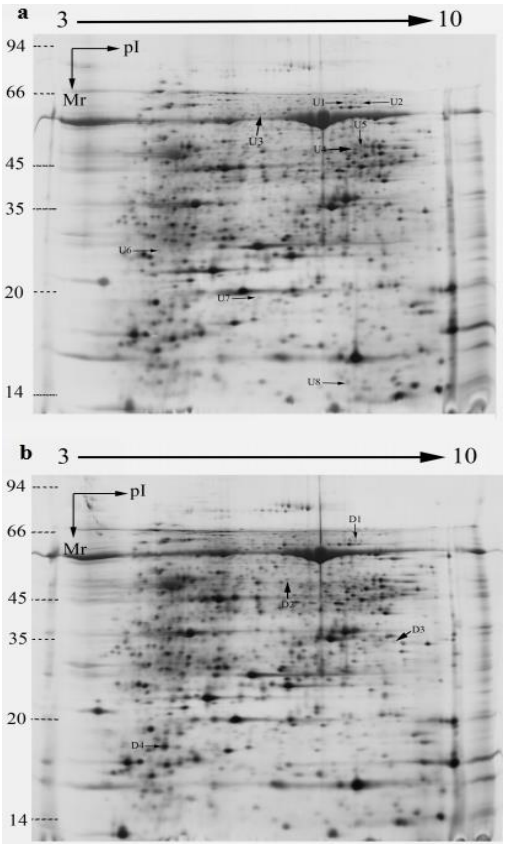

Fig. 10: Representative 2-DE maps of total proteins in *C_4-pepc* transgenic rice under low nitrogen. **a**: PC; **b**: WT. Differentially expressed protein spots identified are indicated with arrows. Map of PC (**a**) shows 8 up-regulated spots (U1-U8). Map of WT (**b**) shows 4 down-regulated spots (D1-D4). The pI extended from pH 3 to 10 and the molecular mass (Mr) from 14 to 94 kDa

and enhanced the leaf N content, which showed a coordinated balance between C/N metabolism in PC under low nitrogen condition. Accordingly, N deficiency increased N assimilation of PC compared to WT and impeded the production of starch or soluble sugars, which in turn induced higher photosynthesis under the increased PEPC activity of PC.

The nitrogen responsive proteins identified in this study could be classified into six functional categories (Table 2). They were involved in some biological processes under low nitrogen stress, including hydrogen peroxide catabolic process, photosynthesis, tricarboxylic acid cycle, amino acid biosynthesis, defense response and translation.

Photosynthesis It has been reported that there were some nitrogen stress-responsive proteins in rice involved in carbon assimilation, including RuBisCo or RuBisCo activase (Hakeem *et al*., 2012). The changes induced by low nitrogen were variable in the abundance of three breakdown products or proteins of Calvin cycle between PC and WT. One spot (U3) was identified as an intact large subunit of RuBisCo and two spots (D2, D3) seemed to be its degradation products because of their smaller observed molecular masses. The intact large subunit of RuBisCo was up-regulated in PC compared with WT under low nitrogen condition, while the degraded fragments were down-regulated. In photosynthesis, RuBisCo is one of the most important enzymes and photosynthesis has a close relationship with the intact RuBisCo rather than its fragments in the abundance and activity (Zhang *et al*., 2010). Therefore, the results indicated that enhanced photosynthesis of N deficient PC was attributed to up-regulation and increased activity of RuBisCo involved in Calvin cycle.

Amino acid biosynthesis Two proteins (U5, U7) showed different expression in PC compared with WT in amino acid biosynthesis under N deficient condition. Phosphoserine aminotransferase and enolase-phosphatase were up-regulated in PC compared with WT. Carbon and nitrogen metabolism can be linked by these two enzymes in plants primarily with amino acids biosynthesis and also took part in photorespiration (Ho *et al*., 1998; Rzewuski *et al*., 2007). Therefore, the up-regulation of two proteins implied that serine and methionine were more and then other amino acids may be produced in N deficient PC.

Hydrogen peroxide catabolic process It has been reported that N deficiency could decrease the activities of antioxidant enzymes, which led to increased oxidative stress in rice (Lin *et al*., 2011). Catalase is one of the antioxidant enzymes, which oxidize toxins (phenols, formic acid, etc.) using hydrogen peroxide (H_2O_2). In this study, CAT (U1, U2) was up-regulated, which was consistent with its higher activity in N deficient PC than in N deficient WT. This result showed that the enzyme might be important in the decrease of oxidative stress under low nitrogen condition. Meanwhile, another antioxidant protein, 2-Cys peroxiredoxin BAS1(U6) was also identified. This protein belongs to one family which is associated with alkyl hydroperoxide reductase and thiol-specific antioxidant. The up-regulation of this protein may be attributed to higher leaf N concentration in N deficient PC since it was involved in conferring NUE with rice genotypes (Hakeem *et al*., 2012). Similar results were obtained in some cultivated rice species under low nitrogen conditions (Kim *et al*., 2009).

Tricarboxylic acid cycle, Defense response, and Translation NAD-dependent isocitrate dehydrogenase (IDH) is one of TCA cycle enzymes that produces 2-OG, which is required in the GS/GOGAT cycle for ammonium assimilation (Gálvez *et al*., 1999). IDH (U4) expression was up-regulated in N deficient PC (Table 2). This indicated that the overexpression of PEPC caused the increase in IDH expression, which led to a higher level of 2-OG (Fig. 5c) in PC under low nitrogen condition. The down-regulation of one pathogenesis-related (PR) protein (D4) was also induced in PC compared with WT under low nitrogen condition. The PR proteins are elicited by pathogen attacks, and there is also some relationship between PR and N

deficiency (Veluthakkal and Dasgupta, 2010; Zhang *et al*., 2016). This result indicated that PC was less sensitive to N deficiency than WT. Meanwhile, the amount of 40S ribosomal protein (U8) in N deficient PC was larger than in N deficient WT, which indicated that low nitrogen might interfere gene expression of PC more than WT.

Conclusion

Our results suggested that the improved low nitrogen tolerance of PC was attributed to elevated PEPC activity, metabolic and proteomic changes under N deficiency. In PC, the increased PEPC activity and *C_4-pepc* gene expression induced higher activities or expressions of C and N assimilation enzymes and contents of organic acids, which suppressed the accumulation of starch or soluble sugars. These responses enhanced the antioxidant enzymes, reducing the inhibition of Pn and retaining higher N content of leaves under low nitrogen condition. A higher abundance of N deficient PC was observed in some proteins involved in photosynthesis, tricarboxylic acid cycle, amino acid biosynthesis, ROS scavenging and translation than of N deficient WT. Further molecular studies will be needed to prove the regulation of PEPC on C/N metabolism in PC under low nitrogen condition. This kind of research may offer clues for improving the C/N balance in rice and be helpful to breed rice with the high photosynthetic capability and great N assimilation efficiency.

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