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Research Note

# Characteristics of photo-oxidation in *phosphoenolpyruvate carboxylase*-transgenic pollen lines of rice

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**Abstract**

The JAAS45 pollen line is a new breeding line with high photosynthetic efficiency and high yield. The aim of this study was to elucidate the photo-oxidative characteristics and other inherited physiological traits of the JAAS45 pollen line of rice (*Oryza sativa* L.) and its parents. The photosynthetic rate, yield components, chlorophyll fluorescence parameters, and antioxidant enzyme activities were measured in leaves of the stable JAAS45 pollen line and its male parent (maize *phosphoenolpyruvate carboxylase*-(*PEPC*-) transgenic rice) and female parent (*Japonica* rice cv. 9516). Under photo-oxidative conditions, the net photosynthetic rate, maximum photochemical efficiency of photosystem II, and photochemical quenching showed smaller decreases in the JAAS45 pollen line than in 9516, its female parent. Non-photochemical quenching was significantly higher in JAAS45 than in 9516 under photo-oxidative conditions, indicating that JAAS45 was more tolerant to photoinhibition and photo-oxidative stress. Higher activities of superoxide dismutase and peroxidase in JAAS45 led to lower accumulation of superoxide radicals, suggesting that JAAS45 has a strong antioxidant capacity. As a result, there was a lower content of malondialdehyde, the product of membrane-lipid peroxidation, in JAAS45 than in 9516. These findings show that JAAS45 is more tolerant than its female parent to photo-oxidation, and that the *PEPC* gene can be transferred to common rice cultivars through germplasm transfer. The introduction of the photosynthetic *PEPC* gene into common rice represents a new pathway for breeding photo-oxidation-tolerant rice with high photosynthetic efficiency.

**Keywords:** phospho*enol*pyruvate carboxylase; chlorophyll fluorescence; photo-oxidation; transgenic rice; photosynthetic rate.
A**bbreviations:** DTT_dithiothreitol; *Fv/Fm*_PSII photochemical efficiency; MDA_malondialdehyde; MDH_NAD malate dehydrogenase; MV_methylviologen; NADPH_nicotinamide adenine dinucleotide phosphate; NTB_2-nitro-5-thiobenzoic acid; PEPC_phospho*enol*pyruvate carboxylase; PEP_phosphoenolpyruvic acid; POD_peroxidase; Pn_photosynthetic rate; PVP_ polyvinylpolypyrrolidone; *qP*_photochemical quenching; $q^N$_non-photochemical quenching; $O_2^{\cdot-}$_ superoxide anion radical; SOD_ superoxide dismutase.

## Introduction

In recent years, the maize gene encoding phospho*enol*pyruvate carboxylase (PEPC), a key enzyme in the $C_4$ photosynthetic pathway, has been introduced into rice, a $C_3$ crop plant (Bandyopadhyay et al., 2007; Zhu et al., 2010). The *PEPC*-transgenic rice lines were shown to have a high photosynthetic capacity (Zhang et al., 2009) and displayed increased tolerance to photo-oxidation under high light intensity (Jiao et al., 2005). The original type of untransformed rice was the *Japonica* rice cultivar "Kitaake", which is grown in northern Japan. This cultivar was unsuitable for cultivation in areas with high light intensity and high temperatures, and had other poor agricultural qualities. The *PEPC*-transgenic rice germplasm was created by crossing a male parent harboring the maize *PEPC* gene with the *Japonica* rice cultivar 9516 as the female parent. Then, the JAAS45 pollen line and H137 pollen lines were screened from the anthers of $F_1$ hybrids by anther culture (Li et al., 2005). The maize $C_4$-specific *PEPC* gene was successfully introduced into the JAAS45 line, and its high level of expression was confirmed by polymerase chain reaction analyses. The photosynthetic characteristics of *PEPC*-transgenic rice could be stably transferred to the hybrid progenies (Ling et al., 2007). Therefore, the *PEPC* gene could be stably inherited in the JAAS45 line.

Phospho*enol*pyruvate carboxylase is a cytosolic enzyme that is widespread among higher plants, and it plays an important role in the response to photo-oxidative stress (Doubnerová and Ryšlavá, 2011). Our research group has shown that introducing the *PEPC* gene into parents of hybrid rice could increase the photosynthetic efficiency of these lines, and reduce photoinhibition damage. Our previous study has shown that the introduction of the maize *PEPC* gene into rice has a variety of photo-protective effects (Jiao et al., 2005). Therefore, using the JAAS45 line and its parents as materials, we systemically surveyed chlorophyll fluorescence parameters and antioxidant indices to explain the inherited physiological properties of *PEPC*-transgenic rice. The results of this study provide theoretical proof for the integration and application of biological technology and general breeding to improve plants' tolerance to photo-oxidation.

## Results and discussion

### *Photosynthetic rate and agronomic performance of JAAS45 pollen line*

The JAAS45 line was obtained from three pollen lines by γ-ray

**Table 1.** Photosynthetic rate and agronomic performance of 9516, JAAS45, PC, and WT.

| Cultivar | Photosynthetic rate ($\mu mol \cdot m^{-2} \cdot s^{-1}$) | No. of panicles per plant | No. of grains per panicle | 1000-grain weight (g) | Grain yield per plant (g) | PEPC activity ($\mu mol.mg^{-1}.h^{-1}$) |
|---|---|---|---|---|---|---|
| JAAS45 | 28.4±1.3 a | 16.1±0.3b | 136.2±6.7a | 29.2±0.8a | 42.1±2.4a | 802.2±39.5b |
| 9516 | 21.2±0.9 b | 12.1±0.3c | 106.4±6.5b | 28.3±0.7a | 39.1±2.6a | 88.4±6.7c |
| PC | 31.4±1.4 a | 20.2±0.4a | 78.3±4.2c | 21.1±0.6b | 30.3±1.8b | 1243.4±51.1a |
| WT | 18.9±0.8b | 20.1±1.3 a | 71.6±4.1 c | 19.3±1.2 b | 30.4±1.9 b | 58.9±4.3c |

Notes: JAAS45, fourth-generation JAAS45 pollen line; PC, PEPC transgenic rice (male parent); 9516, *Japonica* rice cv. 9516 (female parent); WT, wild-type rice (*Japonica* rice "Kitaake"). Values are mean ± standard deviation ($n$ = 5). Values followed by different letters are significantly different ($P < 0.05$, Tukey's protected least significant difference test).

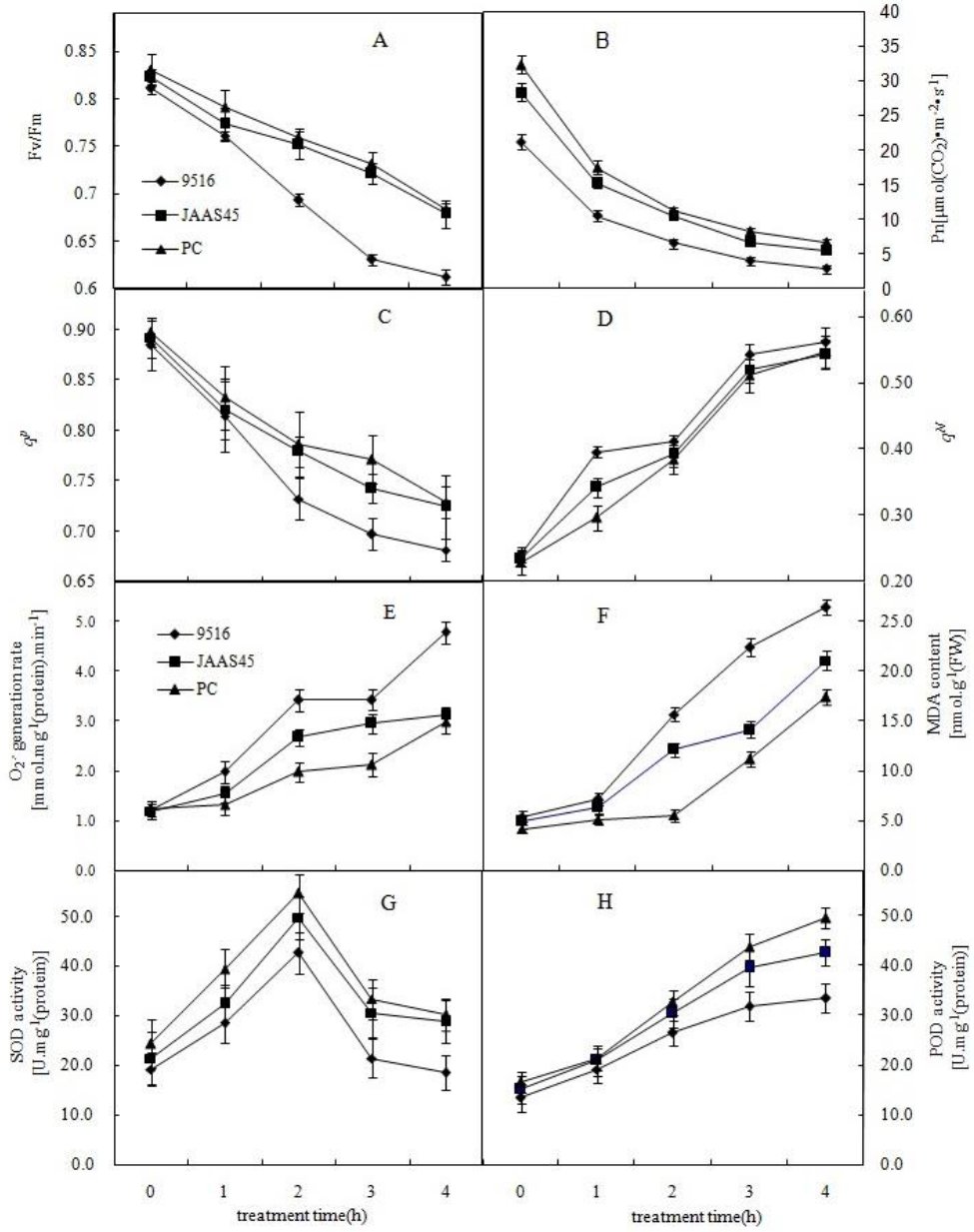


**Fig 1.** Changes in photosynthetic parameters and antioxidant indices in 9516 (female parent), JAAS45 line, and (*PC* male parent) under photo-oxidative conditions. A. Photochemical efficiency of photosystem II (*Fv/Fm*); B. Net photosynthetic rate (Pn); C. Photochemical quenching (*qP*); D. Non-photochemical quenching (*qN*); E. Superoxide ($O_2^{\cdot-}$) generation rate; F. Malondialdehyde (MDA) content; G. Superoxide dismutase (SOD) activity; H. Peroxidase (POD) activity. Values shown are mean ± standard error ($n$ = 5).

radiation and anther culture (Li et al., 2005). As shown in Table 1, the photosynthetic efficiency and the yield per plant were higher in JAAS45 than in its female parent, 9516. This result suggested that the JAAS45 pollen line is a new rice genotype with higher photosynthetic efficiency and yield than those of its female parent.

***Chlorophyll fluorescence parameters and antioxidant indices in JAAS45 pollen line***

During a photo-oxidation treatment, the PSII photochemical efficiency (*Fv/Fm*) (Fig. 1A), photosynthetic rate (Pn) (Fig. 1B), photochemical quenching (*qP*) (Fig. 1C), and non-photochemical quenching ($q^N$) (Fig. 1D) in the JAAS45 line were more similar to those of its male parent, *PC*, than to those of its female parent, 9516. After a 4-h treatment with methylviologen (MV), this trend was much more evident. These results suggested that in JAAS45, more light energy is converted into chemical energy and the excess light energy is released via thermal energy dissipation, resulting in alleviation of photo-damage. The super oxide anion ($O_2^{\cdot-}$) production rate and malonyldiadehyde (MDA) content (a marker of membrane peroxidation) were lower in JAAS45 than in the female parent 9516 during the photo-oxidative treatment (Fig. 1 E,F). During this photo-oxidative treatment, superoxide dismutase (SOD) activity peaked at 2 h and then decreased gradually, while peroxidase (POD) activity increased gradually during the treatment (Fig. 1 G,H). Previous reports have suggested that $C_4$ enzymes function not as photosynthetic enzymes, but as stress-responsive enzymes induced by wounding, low oxygen, low temperature, and salinity (Doubnerová and Ryšlavá, 2011). However, PEPC in rice was shown to be induced under oxidative stress (Jiao et al., 2005), perhaps to compensate for stress-induced photo-damage. The results of the present study showed that the JAAS45 pollen line is more tolerant than its female parent to photo-oxidative stress, and that the *PEPC* gene inherited from its male parent (PC transgenic rice) might have enhanced its oxygen scavenging system. Under high light intensity and high temperature conditions, $C_4$ plants have better $CO_2$-concentrating mechanisms and a greater antioxidant ability than those of $C_3$ plants (Lopes et al., 2011). A previous study showed that stress affected the accumulation of *PEPC* transcripts and PEPC protein in a $C_3$ plant (Doubnerová and Ryšlavá, 2011). Maize genes encoding key components of the $C_4$ photosynthetic pathway have been introduced successfully into rice, a $C_3$ crop plant (Matsuoka et al., 2000). High levels of PEPC expression in rice were shown to improve $CO_2$ assimilation under strong or adverse irradiance conditions (Hatzig et al., 2010), thus alleviating photoinhibition and increasing photosynthetic adaptation. The *PEPC* gene also activated or induced activities of key antioxidant enzymes, including SOD and POD (Zhang et al., 2009). In the present study, the higher SOD and POD activities in the JAAS45 pollen line led to decreased accumulation of active oxygen species. Our data show that introduction of the maize *PEPC* gene into rice produced a variety of photo-protective effects. The JAAS45 pollen line also showed higher PS II photochemical efficiency (Fig. 1A) and lower $O_2^{\cdot-}$ production and MDA content (Fig. 1E, F) than those of the female parent. Therefore, compared with its female parent 9516, the JAAS45 pollen line has higher tolerance to photo-inhibition/photo-oxidation under strong-light and high-temperature conditions, and its photosynthetic physiological characteristics are more similar to those of its male parent (*PEPC*-transgenic rice).

**Materials and Methods**

***Plant materials and analysis of yield components***

Using an *Agrobacterium tumefaciens*-mediated gene transfer system, *PEPC*-transgenic rice plants were obtained by transforming an intact maize gene encoding $C_4$-specific PEPC (GenBank accession: E17154) into the *Japonica* rice (*O. sativa L.*) cultivar "Kitaake". The plant materials used in the present study consisted of PEPC transgenic rice germplasm (PC), Chinese common *Japonica* rice (*O. sativa* L.) cv. 9516, and the JAAS45 pollen line. The stable JAAS45 pollen line was obtained as follows (Li et al., 2005): $F_1$ hybrids were obtained by crossing *PEPC*-transgenic rice as the male parent with 9516 as the female parent. Progenies were obtained by anther culture from the anthers of $F_1$ hybrids, and the haploid and polyploid plants were removed. The diploid plants were selected by examining materials under a microscope. Finally, the JAAS45 pollen line of pure-bred diploid plants was obtained through systematic reselection and re-identification. Seeds of *PEPC*-transgenic rice germplasm, cv. 9516, and the JAAS45 pollen line were grown in a net-door room in Nanjing, China during May-August in 2012 and 2013. Each pot contained five hills, with one seedling per hill. Plants were grown in soil and were watered and fertilized using conventional methods. The rice was harvested at maturity and dried for analyses of yield components.

***Assay of PEPC activity***

The activity of PEPC was assayed according to the methods of Gonzales et al. (1984) and Ku et al. (1999). Approximately 0.25 g leaf tissue was harvested from flag leaves at the heading stage in the light and quickly ground in 1.5 $cm^3$ extraction buffer [50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 2% (w/v) insoluble polyvinyl-polypyrrolidone (PVP), and 10% glycerol]. After total maceration, the crude extract was centrifuged at 13 000 × *g* for 10 min at 4°C, and the supernatant was used immediately for assays of various $C_4$ enzymes. The activity of PEPC was assayed spectrophotometrically at room temperature (30°C) in a mixture containing 50 mM Hepes-KOH pH 8.0, 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 1.5 units NAD-malate dehydrogenase (MDH), 0.2 mM nicotinamide adenine dinucleotide phosphate (NADH), and 20–50 $mm^3$ enzyme extract. The reaction was started by adding phosphoenolpyruvic acid (PEP) to a final concentration of 2 mM. The change in NADH concentration was monitored by measuring the change in absorbance at 340 nm.

***Photoinhibition treatment***

The method of Ling et al. (2006) was used to produce photo-oxidative stress in leaves. The photo-oxidative reagent containing 1.5 $mmol \cdot L^{-1}$ MV with 1% v/v Tween-80 was smeared on the upper surface of intact flag leaves. Distilled water containing 1 % v/v Tween-80 was used as a control. The plants were kept in a room with weak light (20–30 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 2 h to allow the solvent to permeate into the leaves. Then, the rice plants were illuminated at 1400 $\mu mol \cdot m^{-2}\ s^{-1}$ for 3 h. The chlorophyll fluorescence parameters and antioxidant indices of rice leaves were determined after this treatment

***Measurement of chlorophyll fluorescence parameters and photosynthetic rate in leaves***

After a 20-min dark adaptation, the chlorophyll fluorescence

parameters of the leaves were measured using an FMS-2 fluorescence meter (Hanstech, Norfolk, UK) according to the methods of Genty et al. (1989). We used the following equations to calculate the chlorophyll fluorescence parameters:
*Fv/Fm*= (*Fm–Fo*)/*Fm*,
where *Fo* is minimum fluorescence (Genty et al., 1989).
*qP* = (*Fm*'*–Fs*)/(*Fm*'*–Fo*),
where *Fm*' is the maximum fluorescence yield after light adaptation and *Fs* is the steady state fluorescence yield (Foyes et al., 1994).
*qN* = (*Fm–Fm*')/(*Fm–Fo*)
The net photosynthetic rate (Pn) of attached leaves under high irradiance (1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in air was measured using a portable photosynthetic gas analyzer (model TPS-2, PP Systems, Hitchin, UK).

***Antioxidant indices***

Superoxide dismutase activity was assayed by the method of Giannopolitis and Ries (1977). One unit of SOD activity was defined as the amount of enzyme that caused a 50% inhibition of the initial rate of 2-nitro-5-thiobenzoic acid (NBT) reduction. Peroxidase activity was assayed by the method of Kochba et al. (1992). One unit of POD activity was defined as an increase of 0.1 absorbance unit per minute. Malonyldiadehyde content was determined according to the method of Heath and Packer (1968), using the following calculation: $\varepsilon_{532-600nm} = 1.55 \times 10^5\ M^{-1}\ cm^{-1}$. The $O_2^{\cdot -}$ production rate was measured according to the method of Alam et al. (2014), using the following calculation: amount of $O_2^{\cdot -}$ produced/reaction time × amount of protein.

***Statistical analysis***

There were five replications (five pots) for all measurements. Photosynthetic rate, agronomic performance, chlorophyll fluorescence parameters, and antioxidant enzyme data were subjected to analysis of variance (completely randomized) to test the significance of differences among lines. Differences were determined by the Tukey's protected least significant difference test ($P < 0.05$).

**Conclusion**

Compared with the female parent 9516, JAAS45 showed higher tolerance to photo-oxidation under strong light and high-temperature conditions, and their photosynthetic physiological characteristics were more similar to those of its male parent than to those of its female parent. The JAAS45 pollen line obtained by anther culture technology provides new options for breeding new cultivars stably expressing the *PEPC* gene from $C_4$ plants. In conclusion, the use of conventional hybridization and biotechnology, that is, crossing *PEPC*-transgenic rice, represents a new pathway to breed improved rice cultivars that are tolerant to photo-oxidation. Such lines may be more suitable for cultivation in regions with high irradiance and high temperatures such as India, Africa, south China, and south America.

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