DOI: 10.1007/s11099-015-0107-4 PHOTOSYNTHETICA 53 (3): 336-341, 2015

Effects of Ca^{2+} and polyethylene glycol on the chlorophyll fluorescence parameters of transgenic *OsCaS* rice (*Oryza sativa* L.)

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Abstract

Ca^{2+} is an important factor mediating many biotic and abiotic stress responses in plants. In this study, we measured the chlorophyll (Chl) fluorescence of transgenic rice with increased or decreased expression of a calcium-sensing receptor (*OsCaS*) gene during water deficit caused by polyethylene glycol to prove our hypothesis that increased Ca^{2+} in combination with increased *OsCaS* could enhance the drought resistance of transgenic rice. Transcript abundance (evaluated by RT-PCR) was significantly lower in *OsCaS* antisense line 766 (AS766) than that in the wild type, while the overexpression line 777 (O777) showed four times higher amount than that in the wild type. Chl fluorescence showed that the photochemical quantum yield of PSII in the light increased due to addition of Ca^{2+} in the O777, but dropped in the AS766. Nonphotochemical quenching increased under stress in both transgenic lines and in the wild type, but less in the O777. Nonregulatory quantum yield of energy dissipation showed no significant change under drought stress. Photochemical quenching was significantly higher in the O777 than those in the AS766 and in the wild type after the Ca^{2+} treatment. In the absence of stress, the electron transport rate (ETR) was significantly higher in the O777 than in both the AS766 and the wild type. In contrast, the ETR of the wild type and both transgenic lines decreased under drought stress, while the effect of polyethylene glycol was partially alleviated by Ca^{2+} addition in the O777. In summary, excitation energy conversion and dissipation by PSII were regulated by Ca^{2+} in the O777. It might partially alleviate the effect of drought stress, whereas addition of Ca^{2+} had no effect in the wild type and the AS766.

Additional key words: calcium-sensing receptor; fluorescence kinetic curves; gene expression; photosynthesis.

Introduction

Water stress can occur when a plant looses more water than that it absorbs, resulting in a reduced cellular water content, osmotic effects, and a disorder of metabolism. Water deficit greatly influences photosynthesis (Li *et al*. 2012, Han *et al.* 2013) by decreasing the photosynthetic rate, stomatal conductance, and Chl content. Moreover, water stress changes the ability of a plant to respond to osmotic stress (Eivazi *et al.* 2007, Habibi *et al*. 2012); plants with high tolerance to water stress can regulate osmotic stress much better than plants with low tolerance. Besides, water stress also leads to overoxidation of cell membranes (Li *et al.* 2005) and increased activity of antioxidant enzymes (Silva *et al.* 2010). All of this reduces the crop yield and motivates efforts to reduce resource waste and increase economic benefits.

Calcium-sensing receptor (CaS) is a chloroplast protein localized in the thylakoid membrane of *Arabidopsis* (Nomura *et al.* 2012). Its N-terminal acidic calcium binding region is exposed to the stromal side of the membrane, and a rhodanese domain is at the C-terminus (Han *et al*. 2003). It has been shown that CaS protein abundance as well as its phosphorylation level increase in response to increasing light intensities. The phosphorylation site has been mapped to Thr380 and is shown to be dependent on the

Received 31 December 2013, *accepted* 12 August 2014.
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Abbreviations: AS766 – *OsCaS* antisense line 766; CaS – calcium-sensing receptor; $[Ca^{2+}]$ – concentration of Ca^{2+}; $[Ca^{2+}]_i$ – concentration of cytosolic Ca^{2+}; $[Ca^{2+}]_o$ – concentration of extracellular Ca^{2+}; ETR – electron transport rate; O777 – *OsCaS* overexpression line 777; PEG – polyethylene glycol; q_P – photochemical quenching; WT – wild type; Y_{NO} – nonregulatory quantum yield of energy dissipation; Y_{NPQ} – nonphotochemical quenching; Y_{PSII} – practical quantum yield of PSII.
Acknowledgements: We thank to Dr. Yikun He of Capital Normal University for helpful discussion, to Dr. Tiegang Lu of Chinese Academy of Agricultural Sciences for donating pCUbi1390 vector, and to Dr. Yaping Fu and Wenzhen Liu of China National Rice Research Institute for help with transformation of rice and planting the experimental materials. We thank to Dr. Michael Deyholos for help with amending the article. This work was supported by National Nature Science Foundation of China (30900771) and Beijing Natural Science Foundation (5102006).

STN8 protein kinase (Vainonen *et al*. 2008). Evidence suggests the involvement of CaS in stress responses and signaling pathways. Insertional mutagenesis of CaS resulted in reduced growth, indicating a significant role for CaS in plant growth and development (Vainonen *et al*. 2008).

CaS in *Oryza sativa* is highly similar to *Arabidopsis* CaS; it can coordinate changes in intracellular and extracellular calcium concentration. Both of these proteins have a rhodanese domain at the C-terminus (Zhao 2007). This domain is also found in phosphatases and a variety of proteins, such as sulfide hydrogenases, and deemed to be stress responsive proteins. We therefore speculate that OsCaS has a similar function as CaS in *Arabidopsis*. Here we investigated the Chl fluorescence kinetics of transgenic *OsCaS* rice in order to understand the relationship between OsCaS mediated signaling and photosynthesis under stress.

Materials and methods

Cultivation and treatment of experiment materials: We constructed *OsCaS* sense and anti-sense expression vector and transferred them into rice by *Agrobacterium*-mediated transformation. *OsCaS* gene was integrated in pCUbi1390 vector, which includes ubiquitin intron and promoter (Zhao 2007). Seeds of *Oryza sativa* L. (wild type, WT) and T_4-generation overexpression line 777 (O777) and antisense line 766 (AS766) were germinated on moist filter paper for 2–3 d at room temperature, then they were transferred to a soil:vermiculite mixture (1:1) and grown until the three-leaf stage. Then 60 uniform and healthy plants of each line were respectively selected and transferred to the Hoagland nutrient solution with 1 and 5 mM calcium or without and continued to grow for one week. After that treatment, stress was imposed by injecting saturated polyethylene glycol-6000 (PEG-6000, *Sigma-Aldrich*) solution to the original solution and made a series of 10% (PEG10) or 15% (PEG15) polyethylene glycol saline treatment solution, using Hoagland nutrient solution as control. Two days after treatment, we used the PEG10 group to examine the Chl fluorescence kinetics. The seedlings of the PEG15 group were treated for 3 d and then their phenotypes were analyzed. Plants were grown under controlled environment in chambers with light intensity of 200 μmol(photon) m^{-2} s^{-1}, relative humidity of 70–75%, and 12 h dark/12 h light cycle at 25°C.

Analysis of *OsCaS* gene expression in transgenic rice: Leaves of plants were selected for analysis of *OsCaS* gene expression after 30 d in soil mixture. Total RNA of rice was isolated by Trizol (*Invitrogen Inc*., USA). Plant tissue was ground in liquid nitrogen, then the powder was rapidly transferred into a centrifuge tube with Trizol, blended, and set stationary for 10 min. The homogenate was centrifuged at 12,000 rpm for 10 min. The aqueous phase was then extracted by chloroform and then centrifuged at 12,000 rpm for 10 min. Then isopropyl alcohol (2:3, v/v) was added to the aqueous phase. After incubation at –20°C, the RNA was centrifuged and harvested at 12,000 rpm for 10 min, washed with 75% ethanol twice and dissolved in 20 μl RNase-free water. The ultraviolet spectrophotometer (*NanoDrop 2000, Thermo Fisher Scientific Inc.*, USA) was used to assess and calculate the purity and concentration of RNA. RNA was transcribed into cDNA following *Takara* (Dalian, China) reverse transcription kit protocol.

For real-time PCR, *PREMIER 5.0* was used to design the primers. The primers were synthesized in *Songon Biotech* (Shanghai, China) as follows:
OsCaS: fw-5'-TGGTCCCTGCTTTCTTTCACTC-3',
OsCaS: rv-5'-CGCCTCGGCTTGTTTCG-3',
Actin: fw-5'-CAGCACATTCCAGGAGAT-3',
Actin: rv-5'-GGCTTAGGATTCTTGGGT-3'.

A total of 20 μl real-time polymerase chain reactions contained: 0.5 μl forward primer, 0.5 μl reverse primer, 2 μl cDNA, 10 μl 2 × *SYBR® Premix Ex Taq™* (*Takara*, Dalian, China). Amplification and detection of dsDNA synthesis were performed under the following conditions: 94°C for 3 min and 40 cycles at 94°C for 20 s, 58°C for 20 s and 72°C for 20 s. Transcript expression was measured on a *Bio-Rad-TY7967* real-time PCR detector (*Bio-Rad Laboratories*,USA), and the data were analyzed using software *IQ5* (*BioRad*, USA).

Determination of Chl fluorescence kinetic parameters was done using *Maxi-Imaging-PAM* (*Walz*, Germany). Leaves were subjected to dark treatment 20 min before determination of Chl fluorescence kinetics, and then exposed to irradiance of 336 μmol(photon) m^{-2} s^{-1}, and the measurements were triggered every 20 s. The curves consisting of 17 time points were used to get stable status of the following parameters: photochemical quantum yield of PSII (Y_{PSII}) (Schreiber *et al*. 2007), photochemical quenching coefficient (q_P), electron transport rate (ETR), nonphotochemical quenching (Y_{NPQ}), and nonregulatory quantum yield of energy dissipation (Y_{NO}) (Maxwell and Johnson 2000).

Data analysis: Analysis of experimental data and generation of tables and graphs were done by *Excel 2007*. All data were the average values of three independent experiments. Multiple analyses of Chl fluorescence were done using *SPSS 13.0*. The significance level was $p<0.05$.

Results

Gene expression of transgenic plants: The extracted RNA was of good integrity and fluorescent real-time PCR analysis showed that the *OsCaS* gene expression was four times higher in the line O777 than that of the WT, while that of the line AS766 was only one quarter of the WT plants (Fig. 1).

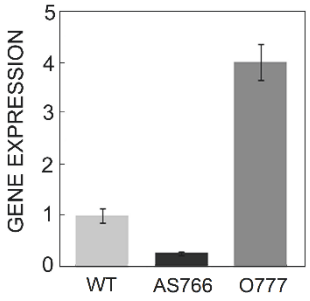

Fig. 1. *OsCaS* gene (calcium sensing receptor gene of rice) expression analysis. AS766 – *OsCaS* antisense line 766, O777 – *OsCaS* overexpression line 777, WT – wild type. Mean ± SD, from three replicates.

Influence of Ca^{2+} and PEG on the Y_{PSII}, Y_{NPQ}, and Y_{NO}: Y_{PSII} represents the actual photosynthetic efficiency; it indicates the primary photochemical efficiency of the PSII reaction centers under the partially closed condition (Murchie *et al.* 2013). The Y_{PSII} of the O777 and AS766 did not show any significant differences in the nutrient solution without Ca^{2+}. After the Ca^{2+} treatment, Y_{PSII} of O777 significantly increased, while that of the WT and AS766 showed no significant change or decreased, respectively (Fig. 2*A*). After the PEG treatment, Y_{PSII} decreased in all plants. Nevertheless, Y_{PSII} could be restored after the Ca^{2+} treatment in the O777, but it remained lower than that without the PEG treatment. However, Y_{PSII} of the WT did not recover after addition of 1 or 5 mM Ca^{2+}. The AS766 showed an opposite trend (Fig. 2*B*).

Y_{NPQ} represents the quantum yield of regulatory energy dissipation and is an important indicator of photoprotection (Moon *et al.* 2011). All the lines did not show significant differences in Y_{NPQ} without added Ca^{2+}. After addition of Ca^{2+}, the O777 showed lower Y_{NPQ} than that in the AS766 and the WT. With the increase of Ca^{2+} concentration, Y_{NPQ} slightly increased in AS766, while the WT showed no obvious difference (Fig. 3*A*). After the treatment with PEG10, Y_{NPQ} of the WT and AS766 increased, but O777 did not change significantly. Y_{NPQ} was lower in the O777 than that of the WT and AS766 either in the presence or in the absence of Ca^{2+} (Fig. 3*B*).

Y_{NO} represents the nonregulatory quantum yield of energy dissipation. When light intensity exceeds the ability of the plant to use the energy, the plant might be injured or is injured if the exposure to light continues (Papageorgiou *et al.* 2011). This is the important indicator of photodamage. In AS766, Y_{NO} increased after the PEG treatment and addition of Ca^{2+} did not change it (Fig. 4*A*). When treated the WT with PEG, Y_{NO} decreased slightly after adding Ca^{2+}. Y_{NO} in O777 slightly decreased only when [Ca^{2+}] was 5 mM (Fig. 4*B*).

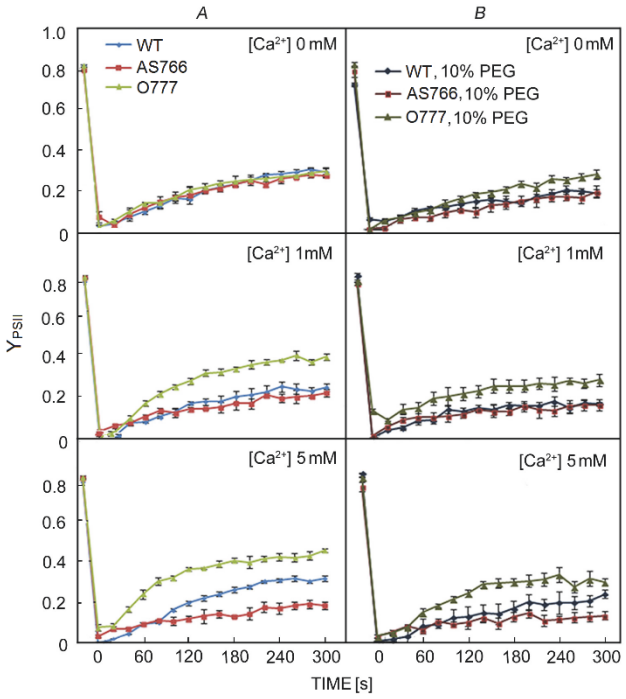

Fig. 2. Comparative analysis of actual photochemical quantum yield of PSII (Y_{PSII}) in *Oryza sativa* under the different concentration of Ca^{2+} (*A*) and under the different concentration of Ca^{2+} and 10% PEG stress (*B*). AS766 – *OsCaS* antisense line 766, O777 – *OsCaS* overexpression line 777, WT – wild type. Mean ± SD, from three replicates.

Influence of Ca^{2+} and PEG on the q_P and ETR: The q_P represents the fluorescence quenching caused by photosynthesis and indicates the level of photosynthetic activity or the fraction of open PSII reaction centers (Luo *et al.* 2013). Without additional Ca^{2+}, the q_P did not show any significant difference in all plants, but after the Ca^{2+} treatment, q_P increased dramatically in O777, WT did not change, and the AS766 showed a decrease (Fig. 5*A*). After the treatment with PEG10, without Ca^{2+}, the q_P decreased in all plants compared to the untreated plants, while after Ca^{2+} addition, q_P increased in the O777 and WT, but it was still lower than that of the untreated group. The AS766 did not show better tolerance to PEG after the addition of calcium (Fig. 5*B*). However, q_P and Y_{PSII} showed the similar trends.

ETR characterizes the transport rate of electrons through PSII. In the nutrient solution without Ca^{2+}, ETR did not show significant differences among the plants. After addition of calcium, ETR increased in the O777. The ETR in the WT decreased in presence of 1 mM Ca^{2+}, but then increased when treated with 5mM Ca^{2+}. The ETR of AS766 decreased after addition of Ca^{2+} (Fig. 6*A*). When seedlings were treated with PEG10 without Ca^{2+}, the ETR decreased in the WT and in both transgenic lines. However, with the addition of Ca^{2+}, ETR increased

as Ca^{2+} increased in the O777 and WT, which alleviated partly the influence of PEG. This effect was not observed in the AS766 (Fig. 6*B*).

Morphological observation: We treated seedlings with PEG to simulate drought stress. We found that adding Ca^{2+} could mitigate the morphological symptoms of PEG-induced stress, especially in the O777. When $[Ca^{2+}]$ increased to 5 mM, the effect from PEG was obviously reduced (Fig. 7*A*). In AS766 and WT, when Ca^{2+} was not present, the seedlings were not damaged as much as in O777. With increasing $[Ca^{2+}]$, all lines improved under drought stress. When $[Ca^{2+}]$ increased to 5 mM, the effect from PEG was obviously reduced in O777 and the growth was generally in agreement with or even better than WT and AS766 (Fig. 7*A,B,C*). We concluded that the O777 was more sensitive to Ca^{2+}. In absence of Ca^{2+}, the O777 appeared seriously damaged, the leaves curled and wilted. But with $[Ca^{2+}]$ increasing, the drought resistant ability of O777 was improved. The plant phenotype proved the better resistance to drought in O777 with increasing Ca^{2+}; Chl fluorescence parameters suggested higher photosynthetic activity of the O777.

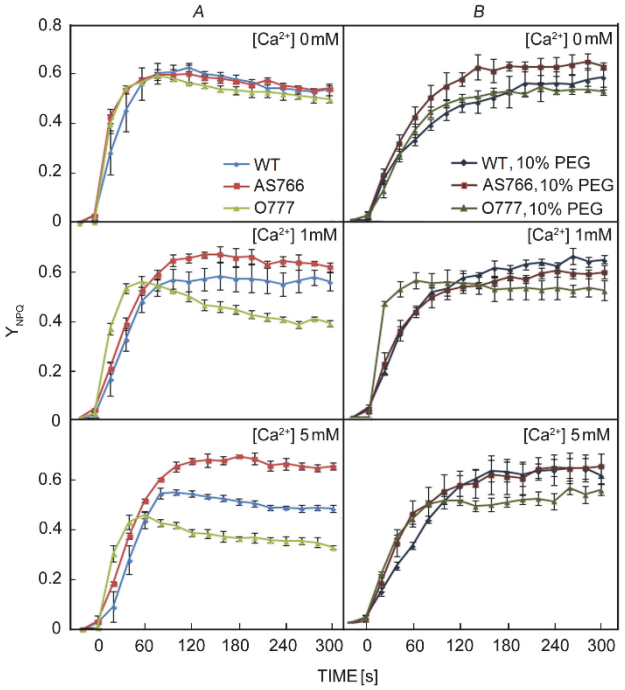

Fig. 3. Comparative analysis of nonphotochemical quenching (Y_{NPQ}) in *Oryza sativa* under the different concentration of Ca^{2+} $[Ca^{2+}]$ (*A*) and under different $[Ca^{2+}]$ and 10% PEG stress (*B*). AS766 – *OsCaS* antisense line 766, O777 – *OsCaS* overexpression line 777, WT – wild type. Mean ± SD, $n = 3$.

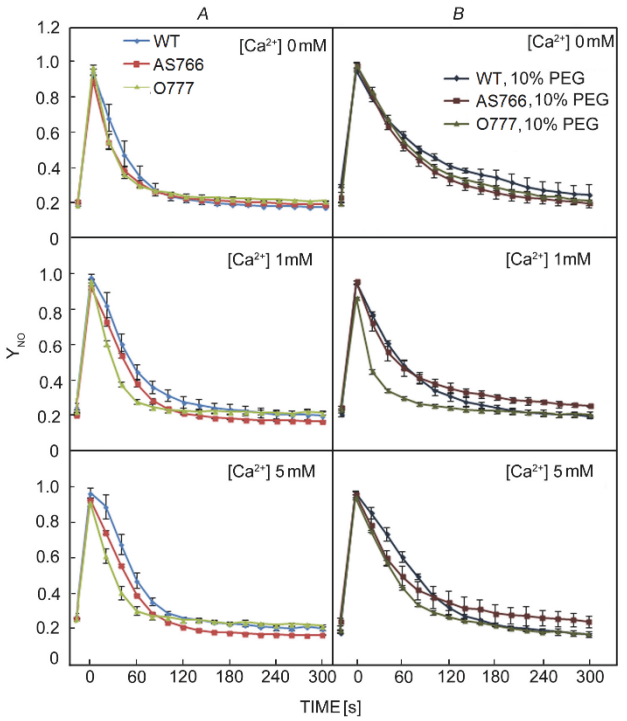

Fig. 4. Comparative analysis of nonregulatory quantum yield of energy dissipation (Y_{NO}) in *Oryza sativa* under different concentration of Ca^{2+} $[Ca^{2+}]$ (*A*) and under different $[Ca^{2+}]$ and 10% PEG stress (*B*). AS766 – *OsCaS* antisense line 766, O777 – *OsCaS* overexpression line 777, WT – wild type. Mean ± SD, $n = 3$.

Discussion

The CaS protein is localized in the thylakoid membrane; it raises the possibility that it may be functionally involved in photosynthesis (Vainonen *et al.* 2008). We obtained the stably inherited *OsCaS* gene overexpression (O777) and antisense (AS766) lines. We confirmed that OsCaS is highly similar to CaS and responds to extracellular $[Ca^{2+}]_o$ resulting in an increase of intracellular $[Ca^{2+}]_i$ (Zhao 2007). Calcium is frequently associated with the stress tolerance (Xu *et al.* 2013, Yang *et al.* 2013). Calcium has been shown to ameliorate the adverse effects of water stress on plants (Jaleel *et al.* 2007) and is involved in signaling antidrought responses (Shao *et al.* 2008). Ca^{2+} signaling is required for the acquisition of drought tolerance or resistance (Cousson *et al.* 2010).

Various reactions in photosynthesis can be studied through Chl fluorescence (Bukhov *et al.* 2004). The influence of stress on photosynthesis can be inferred through changes in Chl fluorescence parameters (Luo *et al.* 2011, Ding *et al.* 2012). The absorbed photons of the PSII reaction center mainly dissipate and are used in three ways: Y_{PSII}, Y_{NPQ}, Y_{NO}, where $Y_{PSII} + Y_{NPQ} + Y_{NO} = 1$ (Papageorgiou *et al.* 2011). The results showed that Y_{PSII} in the O777 increased but it was reduced in the AS766 with the Ca^{2+} increasing under both normal and drought stress conditions. Furthermore, when plants were under drought stress, theY_{PSII} was reduced, but that of O777 could be

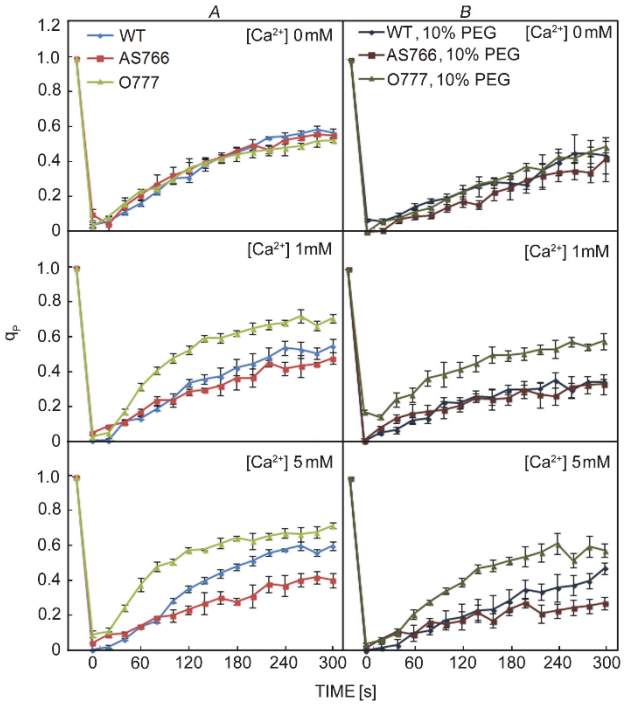

Fig. 5. Comparative analysis of photochemical quenching (q_P) in *Oryza sativa* under different concentration of Ca^{2+} [Ca^{2+}] (*A*) and under different [Ca^{2+}]and 10% PEG stress (*B*). AS766 – *OsCaS* antisense line 766, O777 – *OsCaS* overexpression line 777, WT – wild type. Mean ± SD, $n = 3$.

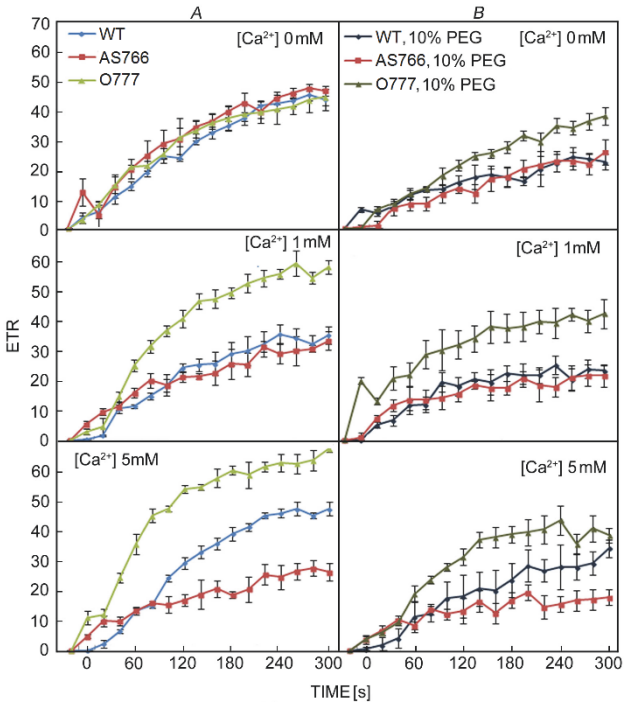

Fig. 6. Electron transport rate (ETR) in *Oryza sativa* under different concentration of Ca^{2+} [Ca^{2+}] (*A*) and under different [Ca^{2+}] and 10% PEG stress (*B*). AS766 – *OsCaS* antisense line 766, O777 – *OsCaS* overexpression line 777, WT – wild type. Mean ± SD, $n = 3$.

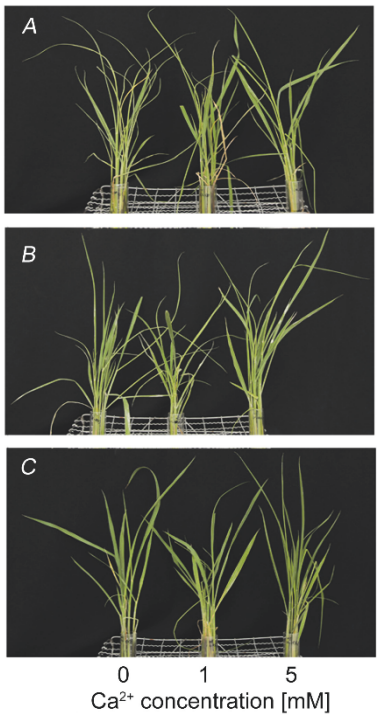

Fig. 7. *Oryza sativa* seedlings treated by 15% PEG for 72 h. Line O777 (*A*), line AS766 (*B*), and wild type (C).

restored partially with Ca^{2+} increasing (Fig. 2*A*,*B*). The inverse trend was observed in Y_{NPQ} and the amplitude of reduction was more significant in O777 (Fig. 3*A*). However, there was not significant change in Y_{NO} after drought treatment (Fig. 4*A*,*B*). The results suggested that in the O777 under drought, Ca^{2+} helped to limit light-induced damage of the PSII reaction center by regulating energy dissipation.

The q_P refers to the activity of light reaction center (Ptushenko *et al.* 2013). We showed that adding Ca^{2+} could significantly improve q_P in the O777. Furthermore, Ca^{2+} could counteract q_P decline caused by PEG in the O777. However, the effect was not obvious in the WT or AS766 lines.

ETR is a reliable indicator of stress on the photosynthetic apparatus. We found that after adding Ca^{2+}, ETR in the O777 was significantly higher than that either of the WT or the AS766. We showed that Ca^{2+} could partially alleviate PSII reaction center closure and inactivation in the O777 under drought stress, while it was not found in the AS766.

In general, the addition of Ca^{2+} could partially alleviate drought damage to the photosynthetic apparatus, especially in the O777. Compared to the WT, O777 maintained photosynthesis in the presence of Ca^{2+} better than either WT or AS766. It was confirmed that OsCaS is highly homologous with CaS in *Arabidopsis*, and they all mediated the changes in intracellular and extracellular calcium concentration (Zhao 2007). Therefore we could conclude that the rice plants overexpressing *OsCaS* were

more sensitive to Ca^{2+}. Ca^{2+} might help the PSII reaction center to stay open and it might thus contribute to improved utilization of light energy. This might increase the resistance to drought stress in agricultural production.

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