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ENHANCED DUCKWEED ALKALI TOLERANCE BY OVERSPRESSION OF SERINE: GLYOXYLATE AMINOTRANSFRASE (ATAGT1)

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Abstract

Alkaline salts are more harmful to plants than neutral salts. Improvement on the alkaline tolerance in plants is important to make full use of the large area of saline-alkaline water. It has been proved that photorespiration pathway provides protection to plants under stress. In this study, *Arabidopsis* serine: glyoxylate aminotransferase (*AtAGT1*)transgenic duckweeds were studied to investigate the alkaline stress tolerance. Enhanced protection from damage to cell membrane was detected in transgenic duckweeds under Na_2CO_3 treatment. Also, in the transgenic duckweed, reactive oxygen species (ROS) accumulation was decreased, and antioxidant enzyme activities were improved with alkaline condition. These results indicated that with the enhancement of photorespiration, antioxidant enzyme activities were improved, leading to the decline of ROS content under alkaline stress. These results also revealed the function of photorespiration enzyme during alkaline stress.

Key words: Alkalinestress; Photorespiration; SGAT; Antioxidant enzyme; ROS; duckweed.

Introduction

There are 0.46 million hm^2saline-alkaline low-lying water in China. Very few aquatic plantscan live in that water, mainly because alkali salt caused conspicuous threat to plants growth and development (Liang *et al.*, 2013). Firstly, alkali salt affected nutrient uptake (Shi *et al.*, 2005; Guo *et al.*, 2015), thus decreasing the dry biomass and changing the morphological features indirectly (Sana *et al.*, 2016). Aslo, alkali salt caused organic acids imbalance. More importantly, the cellular pH stability was seriously disrupted, leading to the comparatively variation in Na^+/K^+ ratio (Khan. *et al.*, 2017), which led to ion homeostasis disturbance (Yang *et al.*, 2007, 2008a). Some reports suggested that a high pH environment also caused direct toxic effects (Yang *et al.*, 2008b). With few aquatic plants survive, these water resources are not effectively utilized (Liang *et al.*, 2013). With enhancement to alkaline tolerance, duckweed, which has a potential to produce liquid biofuel, can be cultivated in the saline-alkaline low-lying water. Therefore, research on the enhancement of alkaline tolerance is important to make full use of these saline-alkaline water resources.

Although photorespiration pathway was supposed as a wasteful way with consuming ATP and releasing CO_2 and NH_3 (Noctor *et al.*, 2002; Hagemann *et al.*, 2016), more and more studies proved that photorespiration was an important pathway to protect plants from stress. Key enzymes of photorespiration have been proved to be involved in abiotic and biotic stress. Ribulose-1, 5-bisphosphate carboxylase/ oxygenase (Rubisco), starting photorespiration and photosynthesis, were up-regulated by salinity (Srivastava *et al.*, 2008), and it was proved to interact with abscisic acid, because Rubisco activation was inhibited by ABA (Galka *et al.*, 2015). Chloroplastic glutamine synthetase (GS-2) was over expressed in rice, and the salt stress tolerance was enhanced (Hoshida *et al.*, 2000). Serine hydroxymethyltransferase (SHMT) protected cell damage during high light and salinity, and also in restricting pathogen-induced cell death stress conditions by minimizing the production of reactive oxygen species (ROS) during pathogen stress (Moreno *et al.*, 2005). Serine: glyoxylate aminotransferases (SGAT) enhanced the resistance against *Pseudoperonospora cubensis* which could cause downy mildew in melon, which was identified as enzymatic resistance (eR) enzyme (Taler *et al.*, 2004). Furthermore, the *Arabidopsis* SGAT enhanced salinity tolerance in *Lemna minor* (Yang *et al.*, 2013).

Photorespiration pathway is important to plants during stress because that photorespiration metabolism not only can produce H_2O_2, but also regulate the intracellular level of ROS. Taler (2004) found that higher glyoxylate aminotransferase (SGT/AGT) activity was accompanied by higher glyoxylic acid oxidase (GO) activities, and GO catalyzed the reaction of glycolic acid to H_2O_2 and glyoxylate, which indicated the involvement of H_2O_2 in this process. Moreno (2005) found that the Arabidopsis mutant *shmt1-1* accumulated more H_2O_2. The exorbitant concentration of ROS, which was considered as the main reason, reduced the mutant anti-biotic and abiotic stress capability. Therefore, the regulation of ROS signaling molecule is an effective entry point when exploring the plant stress resistant genes. Alkaline salts are more destructive to plants than neutral salts. However, few studies showed the influence of photorespiration enzyme and H_2O_2 to alkaline tolerance.

Duckweed is a wildly distributed floating plant (Landolt, 1986), which can survive in polluted water. With the biomass doubling in 2-3 days, the most quickly growth rate in flowering plants (Landolt & Kandeler, 1987), duckweeds are ideal plants to produce liquid biofuel (Zhao *et al.*, 2012). Few researches were done to study the function of photorespiration enzyme during alkaline stress. In this research, *AtAGT1* transgenic duckweed was applied to study the contribution of SGAT to duckweed during alkaline stress. With the enhancement

of alkaline tolerance, the transgenic duckweed can be cultivated in the saline-alkaline low-lying water, which can make full use of these water resources.

Materials and Methods

Plant condition and Na_2CO_3 addition: The duckweeds (*Lemna turionifera 5511*) were collected from a Lake in Tianjin, China, and cultured as described as Yang *et al.* (2013) in a sterile environment at 24°C with a light period for 16 h light: 8 h dark, with a light intensity of 80 $\mu mol \cdot m^{-2}\ s^{-1}$. Alkali water treatments of different Na_2CO_3 concentrations (12.5, 25, 50 mM) were used to cultivate the stock fronds for 6 h and 24 h, respectively. Then the damage of leaf cell, chlorophyll content, H_2O_2 production, antioxidant enzyme levels of fronds, and MDA accumulation were analyzed.

Analysis of cell viability of leaves: The Evans Blue method was adopted to determine the damage of leaf cell visually. Duckweed fronds were incubated in 0.25% (W/V) Evans Blue Solution for 24 h , then different groups of leaves were conducted by 75% alcohol and 25% acetate at room temperature for 2 h. Evans Blue-stained fronds were photographed using microscope (Leica DFC450C, DM5000).

DAB staining: Duckweeds were treated with 3,3-diaminobenzidine (DAB) day at the concentration of 1 $mg.mL^{-1}$for three minutes. Then leaves were decolourated with 75% alcohol and 25% acetate. The H_2O_2 production was determined visually by the brown color on the leaves. The stained duckweeds were photographedusing microscope (Leica DFC450C, DM5000).

Extraction of enzymes: Duckweed leaves (500 mg) were grounded at 4ºC after liquid nitrogen freezing in 2.4 ml 0.05 M KH_2PO_4/K_2HPO_4 buffer (PBS, pH 7.4) with 0.15 $g.L^{-1}$dithiothreitol (DTT) (Yang *et al.,* 2013; Havir & McHale, 1988). Duckweed homogenate was obtained by centrifuged at 13600 rpm, 5 min, 4°C. Further centrifuge of 13600 rpm were done with 20 min for the supermatant. Subsequently, the enzyme extraction was stored at -80°C until being used for antioxidant enzyme and MDA content assays.

Antioxidant enzymes assay: After the treatment of 6 h and 24 h respectively, superoxide dismutase (SOD) activity was researched by principle that the superoxide dismutase can inhibit reduction of the nitroblue tetrazolium (NBT) under the light (Beauchamp and Fridovich 1971). Reaction system was comprised of 0.05M PBS (1.5 ml, pH 7.4), enzyme extraction (0.3 ml), 0.75 mM NBT (0.3 ml), 0.1 mM ethylenediaminetetraacetic acid (EDTA) 0.3 ml, and. 0.13 M methionine (1.5 ml). The solution was put in the incubator for 20 min, at 80 $\mu mol \cdot m^{-2}\ s^{-1}$ light intensity. SOD activity was measured by the inhibition of NBT reduction which was monitored at 560 nm (Duman *et al.,* 2010).

CAT activity was investigated by observing the change of absorbance at 240 nm on account of H_2O_2 consumption with ultraviolet spectrophotometry (Beers & Sizer, 1952).

POD activity was assayed by the measure of guaiacol method described as Maehly (1955), which was determined by evaluating an oxidation rate of guaiacol in the presence of H_2O_2 at 470 nm.

Determination of MDA content: MDA content was measured with thiobarbituric acid in both acidic and high-temperate situation. The 180 µL extraction was added with 2 ml 0.05 $g \cdot L^{-1}$ TBA, and then treated with boiling water for 15 min (Dhindsa & Matowe, 1981). Then the reaction was immediately stopped for instant cooling. Finally, the absorbance was recorded at 450, 532, and 600 nm. MDA content: 6.45 × (A532 - A600) - 0.56 × A450.

Results

Improved tolerance to alkaline in *AtAGT1* overexpression duckweed: The wild type (WT) duckweed and *AtAGT1* overexpression (OE) duckweed were cultured in liquid medium added with 0, 12.5, 25, 50 mM Na_2CO_3 for 24 h, the fronds of WT showed chorisisphonotype, however, the transgenic duckweeds were greener than the WT duckweeds (Fig. 1). That suggested SGAT enhanced duckweed resistance during alkaline stress.

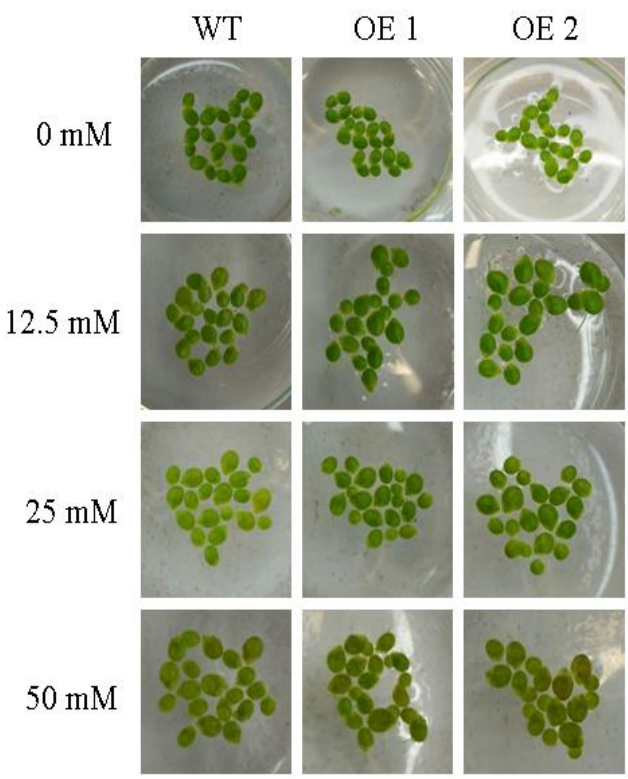

Fig. 1. The phenotype of *AtAGT1* transgenic fronds and WT fronds with addition of different Na_2CO_3 stress for 24 h. OE1–OE2, *AtAGT1* overexpression transgenic lines.WT, wide type duckweeds.

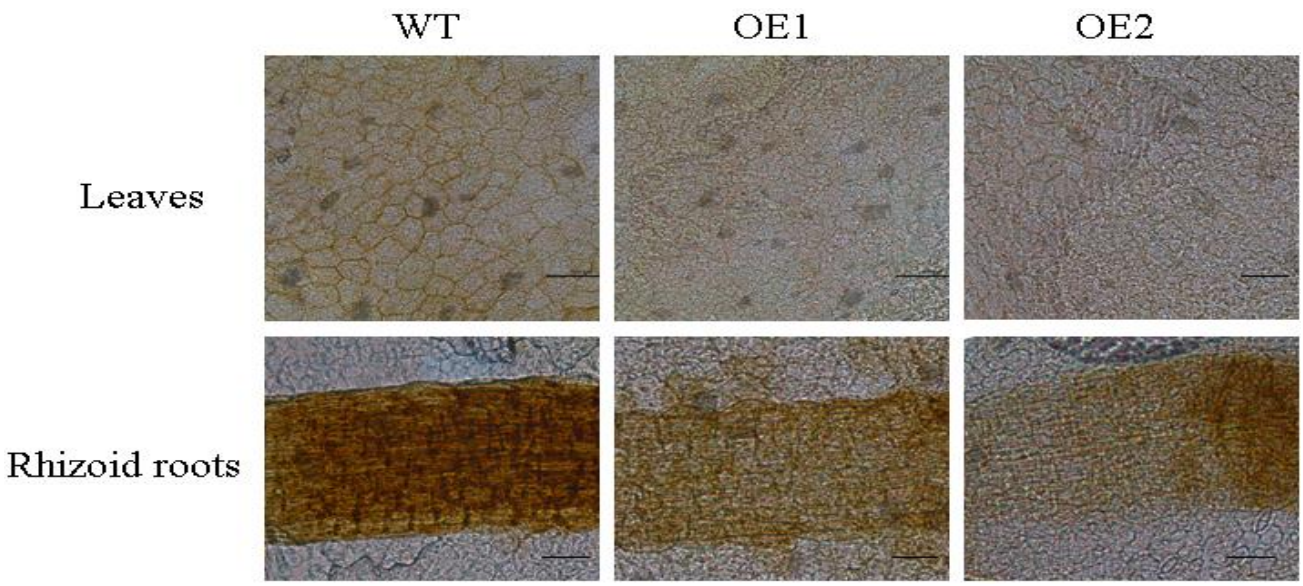

Fig. 2. H_2O_2 content stained by DAB staining in the fronds of the transgenic fronds and WT fronds cultured under 50 mM Na_2CO_3 stress. Scale bars, 50 μm. Pictures are representative of no less than 4 independent experiments with more than 24 fronds in each experimental group.

Decreased reactive oxygen species (ROS) accumulation and improved antioxidant enzyme activity: ROS were stimulated during abiotic or biotic stress. H_2O_2 production in WT and transgenic duckweed under alkaline stress were detected by DAB staining. The result described as Fig. 2 showed that H_2O_2 content in WT duckweed, especially in rhizoid roots, was sharply stimulated under 50 mM Na_2CO_3stress for 24 h. However, in transgenic duckweeds, the H_2O_2 content was obviously decreased in transgenic duckweeds, particularly in the rhizoid roots. That result suggests that SGAT protected transgenic duckweed from ROS burst during alkaline stress.

SOD activities in OE1 and OE2 plants were enhanced slightly (from 43.0 to 50.2 $U \cdot g^{-1}$ (FW), and from 49.1 to 51.4 $U \cdot g^{-1}$ (FW), respectively) with 50 mM Na_2CO_3 treatment for 6 h, while SOD activity in WT plants decreased significantly (from 43.6 to 30.6 $U \cdot g^{-1}$ (FW)). Under 24 h 50 mM Na_2CO_3 treatment, SOD activity in WT duckweed was further reduced to 28.8 $U \cdot g^{-1}$ (FW), 10.5 $U \cdot g^{-1}$ (FW) lower than OE1 duckweed, and 17.5 $U \cdot g^{-1}$ (FW) lower than OE2 duckweed (Fig. 3). The transgenic duckweed showed higher SOD activity compared to WT SOD activity under alkaline stress, indicating that SGAT protected SOD during alkaline condition.

With increasing time of alkali treatment, as shown in Fig. 4, the POD activities in OE plants gradually increased, while POD activity in WT plants first increased in 6 h and then decreased sharply. As a result of 50 mM Na_2CO_3 treatment for 24 h, the transgenic OE1 and OE2 duckweed were 3.04 and 3.24 $U \cdot g^{-1}$(FW), approximately 2 times than the POD activity(1.46 $U \cdot g^{-1}$(FW)). These results imply that *AtAGT1* transgenic duckweed improved POD activity under 50 mM Na_2CO_3 treatment to protect duckweed from peroxide.

The CAT activity in WT and transgenic OE plants was significantly enhanced during alkali treatment for 6 h, suggesting that CAT activity was improved firstly to protect plants from H_2O_2 stress during 50 mM Na_2CO_3 treatment. However, The CAT activity in WT and transgenic OE plants showed remarkable difference during alkali treatment for 24 h. In comparison with the CAT activity in WT plants (11.27 $U \cdot g^{-1}$ (FW)), the CAT activity in OE2 plants was 35.40 $U \cdot g^{-1}$ (FW), three times than the activity in WT plants (Fig. 5). These results explained why the H_2O_2 content was obviously decreased in transgenic duckweeds with 50 mM Na_2CO_3 treatment for 24 h.

Protected cell membrane integrity in transgenic duckweed during alkaline stress: Alkaline stress induced cell membrane damage was tested by Evans blue method. The results showed that leaves and rhizoid roots of WT and transgenic OE duckweed were stained to blue with 50 mM Na_2CO_3stress for 24 h, and WT duckweed were slightly strongly stained compared to the transgenic duckweed, which indicated that alkaline stress caused more damage to cell membrane in WT duckweed (Fig. 6). These result suggested that the transgenic duckweed protected cell membrane in a certain extent during alkaline stress.

The MDA content of WT and transgenic duckweed under alkaline stress were detected (Fig. 7). The MDA content of duckweed tended to be higher with higher alkaline concentrations. In addition, its content in WT duckweed decreased dramatically with the addition of 50 mM Na_2CO_3 stress for 6 h and 24 h (55.3 and 68.8 $\mu mol \cdot g^{-1}$ (FW), respectively). However, the MDA contents in transgenic OE1 and OE2 increased slightly with the treatment of 50 mM Na_2CO_3 stress for 6 h (19.1 and 31.4 $\mu mol \cdot g^{-1}$ (FW), respectively) and 24 h (13.3 and 28.9 $\mu mol \cdot g^{-1}$ (FW) for OE1 and OE2 respectively), which were significantly lower than the WT duckweed, suggesting that transgenic duckweed protected cell membranes from membranous lipid peroxidation damage.

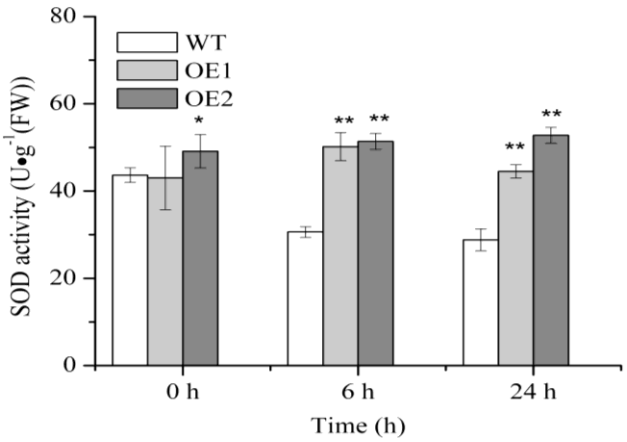

Fig. 3. SOD activity in the transgenic duckweeds and WT plants cultured with 50 mM Na_2CO_3 for 6 h and 24 h. Standard deviations (±SD) were marked by error bars. *Asterisk* (**p<0.01, *p<0.05) presents significant differences from the WT treatments.

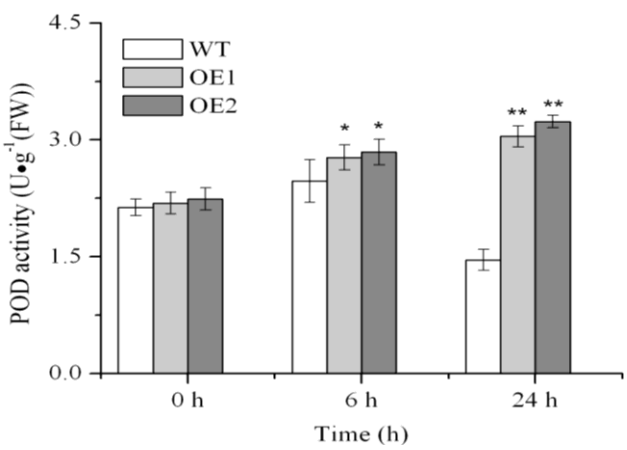

Fig. 4. POD activity in the transgenic duckweeds and WT plants cultured with 50 mM Na_2CO_3 for 6 h and 24 h. Standard deviations (±SD) were marked by error bars. *Asterisks* (**p<0.01, *p<0.05) presents significant differnce from the WT treatments.

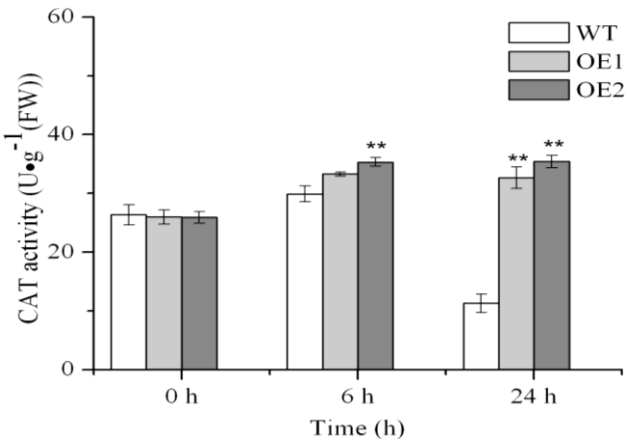

Fig. 5. CAT activity in the transgenic duckweeds and WT plants cultured with 50 mM Na_2CO_3 for 6 h and 24 h. Standard deviations (±SD) were marked by error bars. *Asterisks* (**p<0.01, *p<0.05) presents significant differnce from the WT treatments.

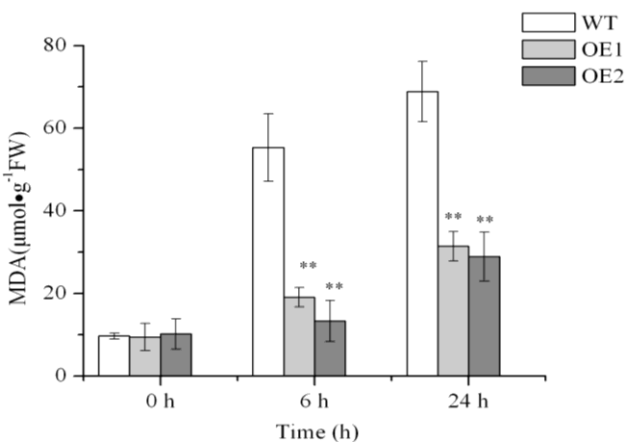

Fig. 6. MDA contents in the transgenic duckweeds and WT plants cultured with 50 mM Na_2CO_3 for 6 h and 24 h. Standard deviations (±SD) were marked by error bars. *Asterisks* (**p<0.01, *p<0.05) presents significant difference from the WT treatments.

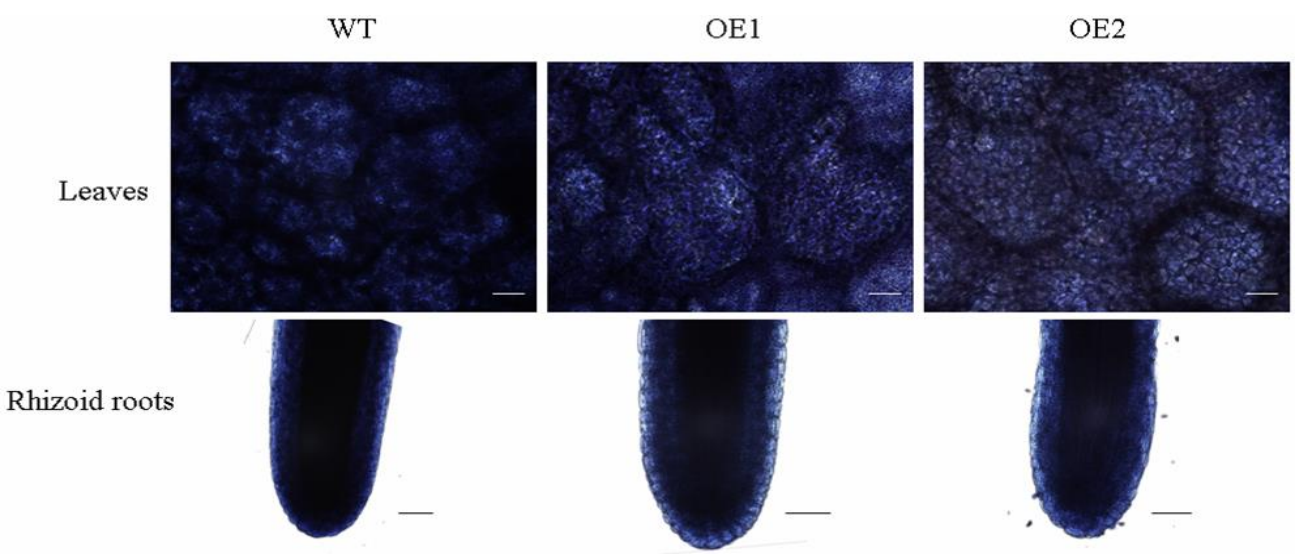

Fig. 7. Cell membrane damage tested by Evans blue staining in transgenic and WT duckweed which treated with 50 mM Na_2CO_3 for 24 h. Scale bars, 50 μm. Pictures are representative of no less than 4 independent experiments and more than 24 fronds in each experimental group.

Discussion

The *AtAGT1* transgenic duckweed was identified here for the study of photorespiration enzyme SGAT, involved in resistance to alkaline stress. SGAT has been proved as enzymatic resistance (eR) gene (Taler *et al.,* 2004), which conferred the resistance to downy mildew in melon. In our previous studies, the function of SGAT during salt stress was investigated by overexpressing Arabidopsis AGT1 (*AtAGT1*), which encode SGAT in duckweed as well. Transgenic duckweed showed enhanced tolerance during salinity stress (Yang *et al.*, 2013). Besides, another enzyme in photorespiration, SHMT, was reported to play an important part in abiotic and biotic stresses such as high light, salinity, and pathogen (Moreno *et al.*, 2005). Not unexpectedly, a number of key enzymes of photorespiration pathway enhanced tolerance to stress in plants.

The present study describes the function of SGAT during alkaline stress in *AtAGT1* transgenic duckweed. The transgenic duckweed showed protection from chlorisis and damage to cell membrane. The content of MDA in *AtAGT1* transgenic duckweed showed significantly declined levels under alkaline stress compared to WT plants. Alkaline stress induced cell membrane damage stained by Evans blue obtained the similar results. These findings suggested that SGAT improved the protection to duckweed during alkaline stress.

Direct evidence for the participation of H_2O_2 in this system was obtained from DAB staining. The transgenic duckweed showed declined H_2O_2 during 50 mM Na_2CO_3 stress for 24 h. These results were further verified by the CAT activity measurements. CAT activity improved with the treatments of alkaline to *AtAGT1* transgenic duckweed, which was three times higher than the WT duckweeds. In *Arabidopsisshmt1-1* mutant, the resistances to biotic and abiotic stress were reduced compare to WT *Arabidopsis*, accompanied by the accumulation of more H_2O_2 (Moreno *et al.,* 2005). Other studies also demonstrated the evidence that the ROS breakout was induced during stress. However, Taler found that GO activity, which response for catalyzing H_2O_2 production, was improved in downy mildew resistance lines (Taler *et al.,* 2004). Jackson & Taylor (1996) found that Reactive Oxygen Intermediate (ROI) production was induced during early stages when the plant was treatedwith stress (Jackson & Taylor 1996). These results suggested that regulation of H_2O_2 and ROS content in plants played a significant role during stress.

This study revealed the relationship among photorespiration, H_2O_2 and alkaline stress. Photorespiration, consuming ATP and releasing CO_2 and NH_3, is not a wasteful way. This pathway has been proved to improve resistance to stress caused by drought, high light (Wingler *et al.,* 2000), disease (Taler *et al.,* 2004), and salinity (Moreno, 2005; Yang *et al.,* 2013). The research in this article provides evidence that photorespiration improves resistance against alkaline media?.

Acknowledgments

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