ENHANCED DUCKWEED ALKALI TOLERANCE BY OVEREXPRESSION OF SERINE: GLYOXYLATE AMINOTRANSFARSE (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 NaCl 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: Alkaline stress; Photorespiration; SGAT; Antioxidant enzyme; ROS; duckweed.

Introduction

There are 0.46 million hm² saline-alkaline low-lying water in China. Very few aquatic plants can live in that water, mainly because alkalai salt caused conspicuous threat to plants growth and development (Liang et al., 2013). Firstly, alkalai 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, alkalai 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 CO₂ and NH₃ (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 absciscic acid, because Rubisco activation was inhibited by ABA (Galka et al., 2015). Chloroplasmic 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 H₂O₂, but also regulate the intracellular level of ROS. Taler (2004) found that higher glyoxylate aminotransferase (SGAT) activity was accompanied by higher glyoxyllic acid oxidase (GO) activities, and GO catalyzed the reaction of glycolic acid to H₂O₂ and glyoxylate, which indicated the involvement of H₂O₂ in this process. Moreno (2005) found that the Arabidopsis mutant shmt1-1 accumulated more H₂O₂. 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 H₂O₂ 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$_2$CO$_3$ 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 μmol·m$^{-2}$·s$^{-1}$. Alkali water treatments of different Na$_2$CO$_3$ 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$_2$O$_2$ 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$_2$O$_2$ production was determined visually by the brown color on the leaves. The stained duckweeds were photographed using microscope (Leica DFC450C, DM5000).

Extraction of enzymes: Duckweeds leaves (500 mg) were grounded at 4°C after liquid nitrogen freezing in 2.4 ml 0.05 M KH$_2$PO$_4$ /K$_2$HPO$_4$ 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 supernatant. 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 μmol·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$_2$O$_2$ 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$_2$O$_2$ 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·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: $6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$.

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$_2$CO$_3$ 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.

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Fig. 1. The phenotype of AtAGT1 transgenic fronds and WT fronds with addition of different Na$_2$CO$_3$ stress for 24 h. OE1–OE2. AtAGT1 overexpression transgenic lines. WT, wide type duckweeds.
Decreased reactive oxygen species (ROS) accumulation and improved antioxidant enzyme activity: ROS were stimulated during abiotic or biotic stress. $\text{H}_2\text{O}_2$ production in WT and transgenic duckweed under alkaline stress were detected by DAB staining. The result described as Fig. 2 showed that $\text{H}_2\text{O}_2$ content in WT duckweed, especially in rhizoid roots, was sharply stimulated under 50 mM $\text{Na}_2\text{CO}_3$ stress for 24 h. However, in transgenic duckweeds, the $\text{H}_2\text{O}_2$ 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·g$^{-1}$ (FW), and from 49.1 to 51.4 U·g$^{-1}$ (FW), respectively) with 50 mM $\text{Na}_2\text{CO}_3$ treatment for 6 h, while SOD activity in WT plants decreased significantly (from 43.6 to 30.6 U·g$^{-1}$ (FW)). Under 24 h 50 mM $\text{Na}_2\text{CO}_3$ treatment, SOD activity in WT duckweed was further reduced to 28.8 U·g$^{-1}$ (FW), 10.5 U·g$^{-1}$ (FW) lower than OE1 duckweed, and 17.5 U·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 $\text{Na}_2\text{CO}_3$ treatment for 24 h, the transgenic OE1 and OE2 duckweed were 3.04 and 3.24 U·g$^{-1}$ (FW), respectively, 2 times than the POD activity (1.46 U·g$^{-1}$ (FW)). These results imply that AtAGTI transgenic duckweed improved POD activity under 50 mM $\text{Na}_2\text{CO}_3$ 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 $\text{H}_2\text{O}_2$ stress during 50 mM $\text{Na}_2\text{CO}_3$ 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·g$^{-1}$ (FW)), the CAT activity in OE2 plants was 35.40 U·g$^{-1}$ (FW), three times than the activity in WT plants (Fig. 5). These results explained why the $\text{H}_2\text{O}_2$ content was obviously decreased in transgenic duckweeds with 50 mM $\text{Na}_2\text{CO}_3$ 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 $\text{Na}_2\text{CO}_3$ stress 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 $\text{Na}_2\text{CO}_3$ stress for 6 h and 24 h (55.3 and 68.8 μmol·g$^{-1}$ (FW), respectively). However, the MDA contents in transgenic OE1 and OE2 increased slightly with the treatment of 50 mM $\text{Na}_2\text{CO}_3$ stress for 6 h (19.1 and 31.4 μmol·g$^{-1}$ (FW), respectively) and 24 h (13.3 and 28.9 μmol·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$_2$CO$_3$ 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$_2$CO$_3$ 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. 5. CAT activity in the transgenic duckweeds and WT plants cultured with 50 mM Na$_2$CO$_3$ 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. 6. MDA contents in the transgenic duckweeds and WT plants cultured with 50 mM Na$_2$CO$_3$ 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$_2$CO$_3$ 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 chlorosis 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 H₂O₂ in this system was obtained from DAB staining. The transgenic duckweed showed declined H₂O₂ during 50 mM Na₂CO₃ 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 Arabidopsis shmt1-1 mutant, the resistances to biotic and abiotic stress were reduced compare to WT Arabidopsis, accompanied by the accumulation of more H₂O₂ (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 H₂O₂ 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 treated with stress (Jackson & Taylor 1996). These results suggested that regulation of H₂O₂ and ROS content in plants played a significant role during stress.

This study revealed the relationship among photorespiration, H₂O₂ and alkaline stress. Photorespiration, consuming ATP and releasing CO₂ and NH₃, 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.

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