ENDOPHYTIC INFECTION PROGRAMS THE ASCORBATE-GLUTATHIONE CYCLE IN RICE (*ORYZA SATIVA* L.) UNDER Na_2CO_3 STRESS

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Abstract. Soil salinization and alkalization have been identified as one of the principal causes of impaired productivity of rice plants worldwide. Endophytes reprogram plant metabolism and elicit beneficial effects on rice growth under salt-alkali stress. This work investigates the potential effects of endophytic infection on the key players in specifically the metabolism, ascorbate-glutathione cycle, in rice seedlings (*Oryza sativa* L.) under Na_2CO_3 stress. Compared to uninfected controls, endophytic infection significantly increased the activities of glutathione reductase and ascorbate peroxidase, the contents of total ascorbic acid, reduced ascorbic acid, total glutathione and reduced glutathione, and the ratios of reduced ascorbic acid/dehydroascorbic acid and reduced glutathione/oxidized glutathione, but decreased the dehydroascorbic acid and oxidized glutathione contents in rice seedlings under Na_2CO_3 stress. In addition, H_2O_2 and proline contents in rice seedlings were significantly decreased by endophytic infection. Taken together, endophytic infection holds great promise to improve the resistance to Na_2CO_3 stress for rice plants and the underlying mechanisms involves modulation of the metabolic intensity of ascorbate-glutathione cycle.
Keywords: *oxidative stress, endophytic fungus, salt-alkali stress, proline*

Introduction

Soil salinization and alkalization have emerged as a significant problem for about 932 million hectares of land worldwide and 100 million hectares in Asia (Wang et al., 2018). The phenomenon of soil salinity and alkalinity often appears spontaneously in nature (Chuamnakthong et al., 2019), and significantly suppresses plant growth leading to a substantial reduction of crop production (Zhang et al., 2017; Wang et al., 2018). Therefore, it is imperative to develop more effective approaches to improve the salt-alkali stress tolerance of plants.

Salt-alkali stress generally correlates with diverse pathophysiological processes such as physiological aridity, ion toxicity, pH homeostasis as well as ionic balance of intracellular in stressed plants (Zhang et al., 2020). In addition to the well-identified processes, there is a growing recognition that the salt-alkali stress often triggers abnormal generation of reactive oxygen species (ROS) in plants. Under favorable conditions, ROS at basal levels performs very essential physiological functions in multiple cell processes (Goraya and Asthir, 2016). However, plants under the salt-alkali stress undergo excessive ROS, which has shown to be detrimental to cell survival and might cause impaired plant growth or even eventual death (Gill and Tuteja, 2010; Koffler et al., 2015). These insights imply that ROS scavenging is critical to increase plant tolerance to salt-alkali stress.

Plants utilize the intrinsic antioxidant defense mechanisms to encounter aberrant ROS (Imahori et al., 2016). Increased activities of antioxidant enzymes have been found in

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plants suffering from the salt-alkali stress, which significantly minimize the untoward effects of excessive ROS on plants (Kasim et al., 2012; Mishra et al., 2013; Chumyam et al., 2017). Indeed, multiple antioxidant metabolic pathways act pivotal parts in the adaptation of plants to environmental stresses. Specifically, the ascorbate-glutathione (AsA-GSH) cycle is well identified to represent the most critical player of antioxidant metabolic systems which can scavenge abnormal ROS (Chumyam et al., 2017). This cycle strongly correlates with the anti-oxidative defense mechanism, and its metabolism intensity has a direct association with the plant tolerance (Ma et al., 2008).

Rice represents an important source of food globally. It exhibits poor salt-alkali tolerance especially in the early seedling period (Li et al., 2014; Sun et al., 2019). As such, soil salinization and alkalization emerge as one of the most common abiotic stress for rice plants, which have largely limited its productivity (Rao et al., 2013; Liang et al., 2014). Our previous studies have established that endophytic infection served as an effective biological tool to improve plant tolerance to the salt-alkali stress, and that multiple key functions were involved in the beneficial effects of endophytes on rice seedlings under Na_2CO_3 stress, such as improved nutrient uptake, photosynthesis and organic acid accumulation (Bu et al., 2012; Li et al., 2017). Since the AsA-GSH cycle has been well identified as the key players of ROS scavenging systems, the aim of our study was to explore whether endophyte-induced benefits involve the AsA-GSH cycle in rice seedlings under salt-alkali stress. This work documented that endophytic infection could modulate AsA-GSH cycle, thereby improving the resistance to salt-alkali stress in rice seedlings. The present results provide an encouraging tool to alleviate Na_2CO_3 stress in salt-alkali sensitive plants.

Materials and methods

All experiments in this study were performed in the Laboratory of Biochemistry and Molecular Biology, School of Life Sciences, Shenyang Normal University, China. Furthermore, all the measurements were determined by UV-visible spectrophotometer (UV-6000PC, Shanghai, China).

Preparation of microorganisms and rice seedlings

The endophytic fungus EF0801 from *Suaeda salsa* that is congeneric to *Sordariomycetes* sp. (99% similarity), was obtained and prepared as our previous studies (Bu et al., 2012; Li et al., 2017). Briefly, EF0801 endophytic strain was cultured at 24 ± 1°C in a 150-mL shaker flask at 180 rpm for 12 days. The fermentation broth generated from this procedure was employed for the treatments of rice seedlings.

Rice seeds (*Oryza sativa* L.) were subjected to sterilization with 2.65% sodium hypochlorite for 10 min, thorough rinse with distilled water and then germination. Following transferring to a 500-mL beaker which contained Hoagland solution, the germinating seeds (100 grains) were cultivated in the growth chamber (80% relative humidity, 16 h/8 h light/dark period, 28°C /26°C day/night, and 3000 lux). After 3 days of growth, rice seedlings were subjected to the treatments of Na_2CO_3 and endophytic infection.

Rice seedlings were randomly assigned into 2 groups: 1) endophyte-uninfected seedlings (E-) and 2) endophyte-infected ones (E+) inoculated with 5% fermentation broth. The seedlings of each group were grown in Hoagland solution containing the following concentrations of Na_2CO_3: 0 mM, 5 mM, 10 mM, 15 mM or 20 mM. In E+

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groups, the endophyte which colonized in the rice seedling roots was more than 90%, but none of colonization in E- groups. Diluted Hoagland solution was replenished to each beaker every day. For further analysis, the seedlings were collected and sampled on the 6th day of the treatments.

Determination of the activities of antioxidant enzymes

Fresh leaves (0.3 g) were homogenized with 50 mM phosphate buffer (pH 7.5, 3 mL) which containing 0.1 mM EDTA and TritonX-100 and 4% (w/v) polyvinylpyrrolidone (PVP-40). Following a 25 min centrifugation at 13,000 × g at 4°C, the supernatants were harvested to analyze the activities of glutathione reductase (GR) and ascorbate peroxidase (APX).

GR activity was monitored at 340 nm in reaction mixture (3 mL) containing 0.1 M Tris-HCl (pH 8.0), 1 mM oxidized glutathione (GSSG), 0.1 mL supernatant and 0.2 mM NADPH. The reaction was initiated by adding NADPH.

APX activity was determined by monitoring the decrease in absorbance at 290 nm. The assay mixture (3 mL) contained 50 mM Hepes-KOH (pH 7.6), 1.0 mM H_2O_2, 0.5 mM reduced AsA and 0.05 mL supernatant. The reaction was initiated by adding H_2O_2.

Determination of the antioxidant contents

Fresh leaves (0.4 g) were homogenized in a pre-cooled mortar and pestle containing ice-cold 5% TCA (4 mL). After a 20 min centrifugation at 13,000 × g at 4°C, the supernatants were harvested for analysing the contents of total ascorbic acid (TAsA), reduced ascorbic acid (AsA), total glutathione (TGSH) and reduced glutathione (GSH).

To determine the TAsA content, 1 mL supernatant was mixed with the reaction solution with the addition of 0.1 M phosphate buffer (pH 7.7, 0.25 mL) and 2 mM dithiothreitol (0.25 mL). After 10 min reaction at room temperature, 10% TCA solution (0.4 mL), 44% phosphoric acid (0.4 mL), 4% 2,2-bipyridine (prepared in 70% ethanol, 0.4 mL) and 3% Fe_2Cl_3 (0.2 mL) were mixed with the reaction solution. After 60 min incubation at 37°C, the absorbance was determined at 525 nm.

To quantify the AsA content, 1 mL supernatant was mixed with 0.1 M phosphate buffer (pH 7.7, 0.5 mL). Following 30 s reaction at room temperature, the next procedure was performed as described in the assays of TAsA content.

To quantify the TGSH content, 1 mL supernatant was mixed with 1 U GR, 0.1 M phosphate buffer (pH 7.7, 0.5 mL), 0.15 mM NADPH (1 mL). After 2 min reaction at room temperature, 0.6 mM DTNB (0.6 mL) was mixed with the reaction buffer. After 5 min incubation in a constant temperature bath (37°C), the absorbance was determined at 412 nm.

To quantify the GSH content, 1 mL supernatant was mixed with 0.1 M phosphate buffer (pH 7.7, 2.5 mL) and 0.6 mM DTNB (0.6 mL). After 5 min incubation in a constant temperature bath (37°C), the absorbance was then measured at 412 nm.

Determination of H_2O_2 content

Fresh leaves (0.5 g) were homogenized with 3 mL acetone. After centrifugation at 13,000 × g for 20 min at 4°C, the supernatants were harvested. Afterwards, 5% sulfuric acid (0.1 mL) and ammonia water (0.2 mL) were added to the 1 mL enzyme solution. Centrifugated at 4000 r/min for 10 min, the precipitation was harvested and washed with

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precooled acetone for 2-3 times. Following dissolution by 2 M sulfuric acid (4 mL), the H_2O_2 content in the precipitation was monitored at 520 nm.

Estimation of proline content

Leaves were (0.5 g) homogenized with 3% sulfosalicylic acid (5 mL), and then heated at 100°C for 10 min. After centrifugation at 3,000 × g for 15 min, the supernatant (2 mL) was mixed with distilled water (2 mL), acetic acid (2 mL) and acid ninhydrin (4 mL), boiled for 1 h and the reaction was stopped by cooling the tubes in ice bath for 5 min. The chromophore formed was extracted with toluene by vigorous shaking. Absorbance of the resulting organic layer was measured at 520 nm by spectrophotometer.

Statistical analysis

In this study, each experiment was independently repeated three times and results from the duplicate experiments were combined for statistical analysis. The data were shown as mean ± standard deviation (SD) from three independent experiments. The statistical analysis between the two groups were performed by two tailed Student's t-test or Mann-Whitney test (Czobor et al., 2017). The statistical difference between multiple groups were conducted using one-way ANOVA or Kruskal-Wallis test (Cheng et al., 2020; Liu and Wang, 2021). All statistical analysis was performed using GraphPad Prism (Version 5) and SPSS 16.0. and an alpha of 0.05 was employed for all tests.

Results

Variations in the growth of rice seedling

Na_2CO_3 stress elicited an obvious growth inhibition of endophyte-uninfected rice seedlings in a concentration-dependent manner. In contrast, endophytic infection effectively blocked the inhibition of growth of rice seedlings under Na_2CO_3 (*Fig. 1*).

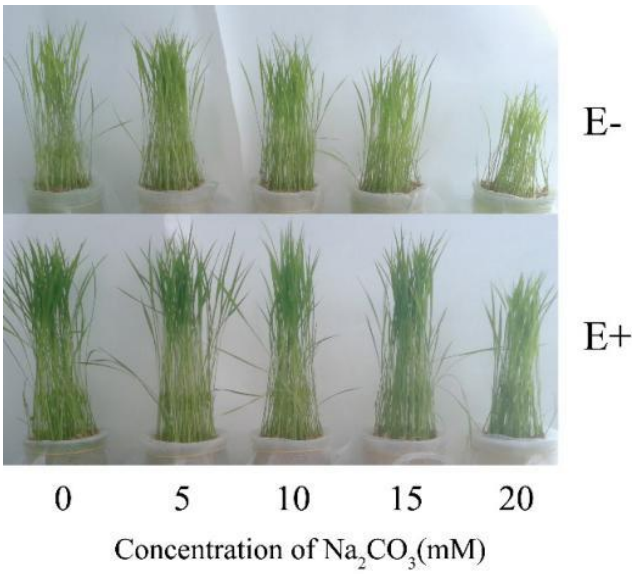

Figure 1. *Variations in the growth of endophyte-uninfected (E-) and -infected (E+) rice seedlings under Na_2CO_3 treatment*

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Variations in the activities of antioxidant enzymes

GR activity in the leaves of endophyte-infected and -uninfected rice seedlings first increased and then decreased with the increasing concentrations of Na_2CO_3 (*Fig. 2a*). When there was no Na_2CO_3 stress, endophytic infection significantly increased the GR activities in the rice seedlings. Rice seedlings with endophytic infection exhibited an obvious increase of GR activity under 5, 10 and 15 mM Na_2CO_3 stress in comparison with uninfected controls, whereas no significant influence under 20 mM Na_2CO_3 stress.

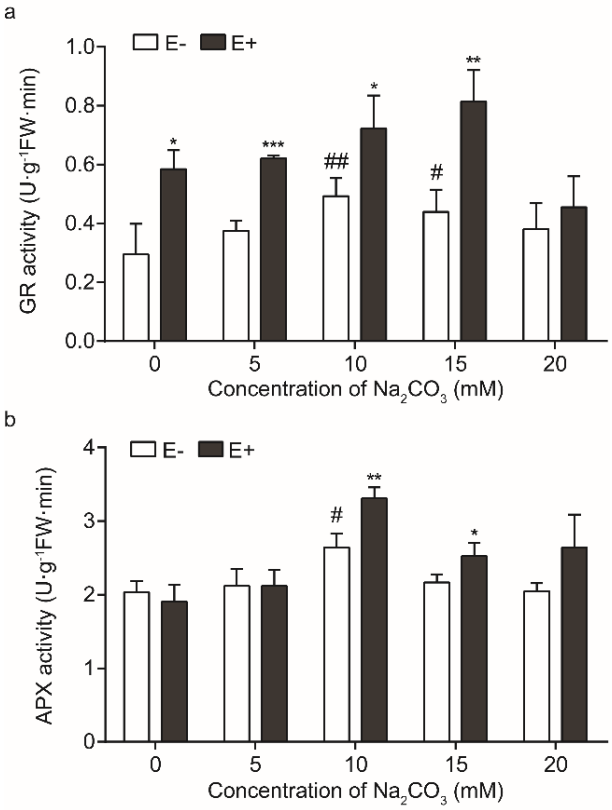

Figure 2. *Variations in the activity of antioxidant enzymes in the AsA-GSH cycle of endophyte-uninfected (E-) and -infected (E+) rice seedlings. Bars indicate standard deviations (n = 3).* $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ *compared with E- 0 mM group by one-way ANOVA (a, b);* $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ *and* $^{***}P \leq 0.001$ *compared with E- group by two-tailed Student's t-test*

APX activity showed a 30.1% increase upon 10 mM Na_2CO_3 stress in comparison with unstressed controls, while endophytic infection had little effects on APX activity in rice seedlings without Na_2CO_3 stress (*Fig. 2b*). Endophytic infection significantly increased APX activities under 10 and 15 mM Na_2CO_3 stress.

Variations in the contents of antioxidants

The effects of Na_2CO_3 stress on the contents of TAsA, TGSH and AsA exhibited a similar pattern (*Fig. 3a, b and c*). The contents of TAsA, AsA and TGSH in the leaves of both endophyte-infected and -uninfected seedlings firstly increased and then declined with the increasing concentrations of Na_2CO_3. Under no Na_2CO_3 stress, infection with the endophyte markedly increased the contents of AsA and TGSH, while TAsA content

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exhibited no significant change. Endophytic infection resulted in a marked increase in AsA and TGSH contents under Na_2CO_3 stress (except AsA under 15 mM Na_2CO_3), as well as the content of TAsA under 5 and 10 mM Na_2CO_3 stress.

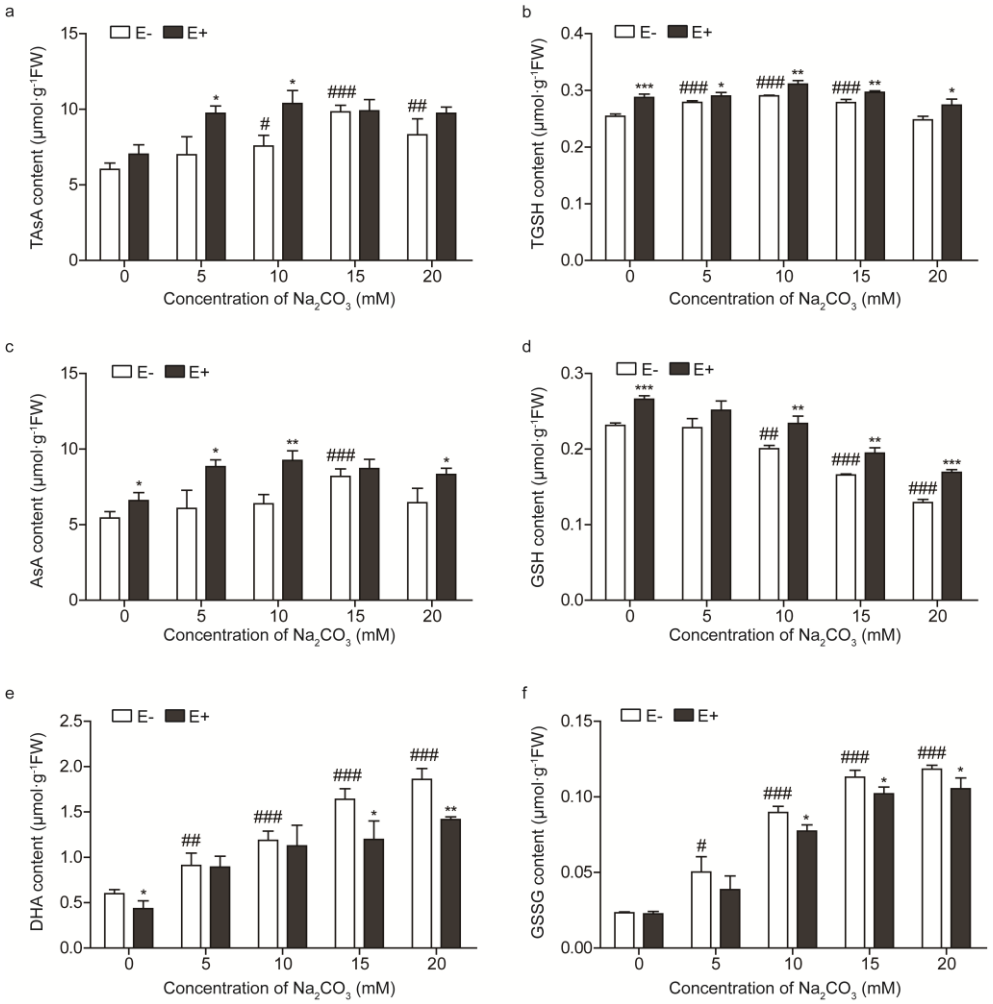

Figure 3. *Variations in the content of antioxidants in the AsA-GSH cycle of endophyte-uninfected (E-) and -infected (E+) rice seedlings. Bars indicate standard deviations (n = 3).* $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ *and* $^{\#\#\#}P \leq 0.001$ *compared with E- 0 mM group by one-way ANOVA (a, b, c, e) or Kruskal-wallis test (d, f);* $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ *and* $^{***}P \leq 0.001$ *compared with E- group by -two-tailed Student's t-test*

The change pattern of the contents of DHA and GSSG under Na_2CO_3 stress was opposite to that of GSH content (*Fig. 3d, e and f*). In both endophyte-infected and -uninfected seedlings, DHA and GSSG contents substantially increased with the increasing concentrations of Na_2CO_3, while GSH content decreased (except under 5 mM Na_2CO_3). Endophyte-infected seedlings displayed a marked increase in GSH contents under 0, 10, 15 and 20 mM Na_2CO_3 stress. When there was no Na_2CO_3 stress, DHA content was decreased by 23.9% with endophytic infection, but no significant difference for GSSG contents were found. Moreover, endophytic infection led to an obvious decrease of DHA contents under 15 and 20 mM Na_2CO_3 stress, as well as the GSSG contents under 10, 15 and 20 mM Na_2CO_3 stress.

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In both endophyte-infected and -uninfected seedlings, AsA/DHA ratio significantly decreased with the increasing concentrations of Na_2CO_3 (*Fig. 4a*). Endophytic infection significantly increased AsA/DHA ratio under 0, 10, 15 and 20 mM Na_2CO_3 stress. A similar pattern was observed for GSH/GSSG ratio (*Fig. 4b)*. Furthermore, endophytic infection resulted in a marked increase in GSH/GSSG ratio under 10, 15 and 20 mM Na_2CO_3 stress.

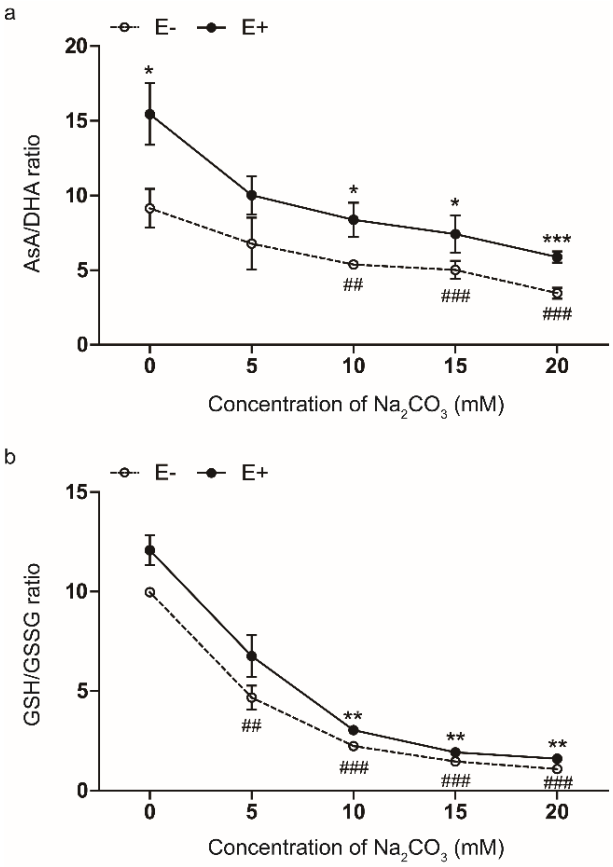

Figure 4. *Variations in the ratios of AsA/DHA and GSH/GSSG of endophyte-uninfected (E-) and -infected (E+) rice seedlings. Bars indicate standard deviations (n = 3).* $^{\#\#}P \leq 0.01$ *and* $^{\#\#\#}P \leq 0.001$ *compared with E- 0 mM group by Kruskal-wallis test (a, b);* $^{*}P \leq 0.05$，$^{**}P \leq 0.01$ *and* $^{***}P \leq 0.001$ *compared with E- group by two-tailed Student's t-test*

Taken together, these findings indicated that the benefit of endophytic infection to plant Na_2CO_3 resistance strongly correlated with the increased metabolic intensity of antioxidant systems (AsA-GSH cycle) (*Fig. 5*).

Variations of H_2O_2 content

The contents of H_2O_2 increased under Na_2CO_3 stress in a concentration-dependent manner in the leaves of both endophyte-infected and -uninfected rice seedlings (*Fig. 6*). Crucially, endophytic infection markedly decreased H_2O_2 contents under 10 and 15 mM Na_2CO_3 stress.

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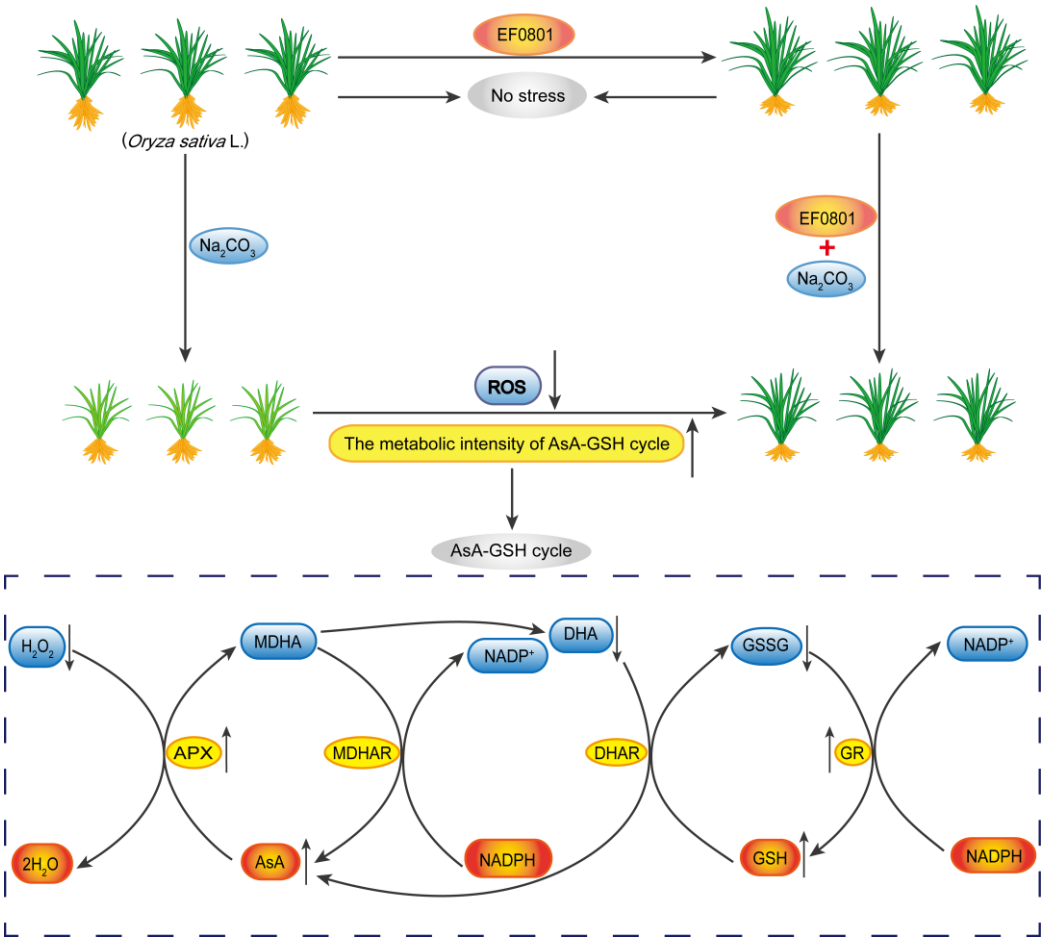

Figure 5. *Proposed model. ROS, Reactive oxygen species; AsA-GSH, Ascorbate-glutathione; H_2O_2, hydrogen peroxide; MDHA, Monodehydroascorbate; DHA, Dehydroascorbic acid; GSSG, Oxidized glutathione; APX, Ascorbate peroxidase; MDHAR, Monodehydroascorbate reductase; DHAR, Dehydroascorbate reductase; GR, Glutathione reductase; AsA, Reduced ascorbic acid; GSH, Reduced glutathione*

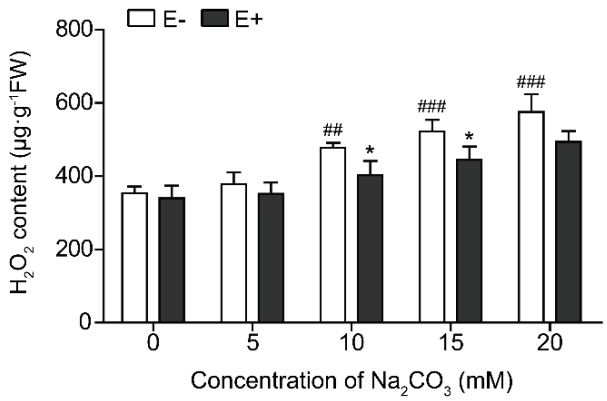

Figure 6. *Variations in the content of H_2O_2 of endophyte-uninfected (E-) and -infected (E+) rice seedlings. Bars indicate standard deviations (n = 3). $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ compared with E- 0 mM group by one-way ANOVA; $^{*}P \leq 0.05$ compared with E- group by two-tailed Student's t-test*

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Variations of proline content

With the increasing concentrations of Na_2CO_3, proline content in both endophyte-infected and -uninfected rice seedlings significantly increased (*Fig. 7*). Endophytic infection significantly decreased the proline content under 10 and 20 mM Na_2CO_3 stress. Thus, we concluded that modulation of proline accumulation seemed to represent another possible defense mechanism of endophyte for the plants against salinity.

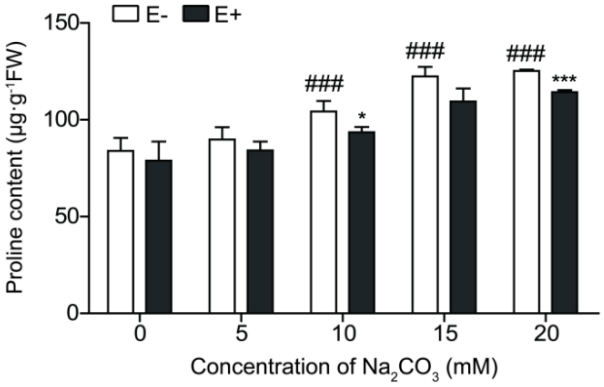

Figure 7. *Variations in the content of proline of endophyte-uninfected (E-) and -infected (E+) rice seedlings. Bars indicate standard deviations (n = 3). $^{###}P \leq 0.001$ compared with E- 0 mM group by one-way ANOVA; $^{*}P \leq 0.05$ and $^{***}P \leq 0.001$ compared with E- group by two-tailed Student's t-test*

Discussion

Salt-alkali represents a main abiotic stress and largely limits crop production worldwide (Zhang et al., 2020). Therefore, designing optimal strategies to enhance plant resistance has been the subject of intense investigation. Our previous studies (Bu et al., 2012; Li et al., 2017) have demonstrated that Na_2CO_3 stress severely inhibited rice growth as evidenced by decreased shoot/root length and dry weight. Crucially, infection with endophyte EF0801 strain in rice seedlings significantly alleviated the growth-inhibition induced by Na_2CO_3 stress. In the present study, we further demonstrated that endophytic infection significantly raised the metabolic power of anti-oxidative defense systems by modulation of AsA-GSH cycle, thereby improving the resistance of rice seedlings to salt-alkali stress (*Fig. 5*).

Endophyte emerges as a fungus or bacterium which frequently lives within plants in their native environments (Gladieux, 2018). Analysis of endophyte-host interactions suggested that fungal endophytes could offer superior advantages of plant growth promotion and stress homeostasis regulation to host plants especially in stress conditions (Mirzahossini et al., 2015). In agreement, endophytic infection in plants had significant capacity to enhance host resistance to multiple stress environments, such as heat (Ismail et al., 2018), drought and salt (Moghaddam et al., 2021), nickel (Mirzahossini et al., 2015) or zinc (Li et al., 2012) or cadmium (Zhang et al., 2010; Ma et al., 2019). Our present findings further demonstrated that endophyte EF0801 strain was able to confer effective resistance to salt-alkali stress.

Excessive ROS represents a frequent event in plants under the salt-alkali stress. The present study demonstrated that Na_2CO_3 stress induced a significant increase of H_2O_2 content in rice seedlings. The anti-oxidative defense system has the abilities to mitigate

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unfavorable impacts of abnormal ROS on plants themselves. The functional activities of antioxidant enzymes GR and APX often serve as credible indicators of the power of anti-oxidative defense systems in plant cells (Ma et al., 2008). Aravind and Prasad (2005) found that the increased activities of GR and APX exerted protective roles in combating Cadmium-induced oxidative stress. Baltruschat et al. (2008) demonstrated that the significant enhancement of APX and GR activities was involved in *P. indica*-induced salt tolerance in roots of salt-stressed barley. In accord with this, our present results showed that increased activities of GR and APX were observed in rice seedlings under Na_2CO_3 stress. After endophyte infection, the activities of GR and APX were further increased. In addition, endophyte-induced tolerance to the salt-alkali stress in rice seedlings might involve modulation of the contents of TAsA, AsA, DHA, TGSH, GSH and GSSG, the main components in the AsA-GSH cycle. Since the ASA-GSH cycle represents key player in ROS scavenging systems, the endophyte-induced increase of metabolic intensity of this cycle may contribute to greater protection from oxidative injury caused by salt-alkali stress.

The AsA/DHA ratio is considered as a well-recognized index to assess the levels of AsA (Li et al., 2010). Higher AsA/DHA ratio generally indicate higher AsA contents, and correlate with the preventation of oxidative injury in plants (Mishra et al., 2013). The increased contents of AsA could effectively reduce ROS induced by salt stress, thereby protecting plants from oxidative attacks (Hasanuzzaman et al., 2014). Shalata et al. (2001) demonstrated that salt-sensitive tomato displayed a significant decrease of the reduced AsA content and an increase of DHA content in response to salt-stress, whereas the salt-resistant plants exhibited the opposite pattern. Furthermore, Baltruschat et al. (2008) demonstrated that salt stress decreased AsA/DHA ratio in endophyte-uninfected barley, while endophytic infection significantly enhanced the ratio of AsA/DHA in plants under saline exposure. In agreement, we observed that Na_2CO_3 stress led to a significant decrease of AsA/DHA ratio, which were effectively restored by endophytic infection in rice seedlings. Na_2CO_3 increased contents of AsA and DHA, but endophyte infection further increased the contents of AsA and decreased the DHA contents.

GSH is shown to be converted from GSSG which could increase the tolerance of plant cells to stress environments (Ma et al., 2019). Aravind and Prasad (2005) reported that Cd-10 μM stress resulted in a decrease of GSH, as well as an increase of GSSG in *Ceratophyllum demersum*. Our results showed that rice plants displayed the similar changes of GSH and GSSG in response to Na_2CO_3 stress. GSH/GSSG ratio represents a crucial index weighing the AsA-GSH cycle metabolism intensity, and has a direct relation with plant tolerance. Selote and Khanna-Chopra (2006) demonstrated that drought stress led to a significant decrease in GSH/GSSG ratio of wheat leaves. Likewise, we found that Na_2CO_3 stress significantly reduced GSH/GSSG ratio of rice leaves. Endophytic infection induced a significant increase of GSH but a decrease of GSSG, thereby effectively restoring GSH/GSSG ratio.

As the most water-soluble amino acid, proline has been shown to accumulate in plants under abiotic stress. However, the physiological significance of proline accumulation still remains a controversial subject. Lutts et al. (1999) found that lower levels of free proline were accumulated in salt-resistant cultivars than salt-sensitive ones in response to salt stress. Furthermore, Garcia et al. (1997) demonstrated that exogenous administration of proline led to exacerbated damages induced by the salt. In the present study, we found that Na_2CO_3 stress induced significant accumulation of proline in rice seedlings, and that endophytic infection led to an obvious reduction of proline contents. Our findings support

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the notion that the accumulation of proline serves as an indicator for stress damage, rather than a component of salt tolerance. Modulation of proline accumulation may represent another possible defense mechanism of endophyte for the plants against Na_2CO_3 stress.

Conclusion

Based on the present study, it is concluded that endophytic infection with the EF0801 strain was capable to protect rice seedlings from Na_2CO_3 stress, and that the benefits of endophyte to the resistance to Na_2CO_3 stress strongly correlated with the increase of the metabolic intensity of AsA-GSH cycle. Yet the molecular basis mediating the effects of endophytic infection on the AsA-GSH cycle requires future efforts. Once its roles and related mechanisms are well evaluated, the EF0801 endophyte will provide new perspectives for alleviating Na_2CO_3 stress in plants with high sensitivity to salt-alkali.

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