Pak. J. Bot., 51(2): 393-399, 2019. DOI: 10.30848/PJB2019-2(3)

LOW IRON LEVEL IMPROVES SALT TOLERENCE BY CHANGING THE REDOX REGULATORY MECHANISMS IN *ARABIDOPSIS THALIANA*

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Abstract

Soil salinization is a critical restrictive causal factor for plant growth. The soluble salts could lead to low crop growth and production; thus, it is very crucial to improve plant salt tolerance. Iron (Fe) is involved in plant growth and development, and many other metabolic pathways. Yet, little research on the relationship between iron content and salt tolerance has been done in previous works. By using a series of Fe gradient treatments, this study explored the role of Fe in *Arabidopsis thaliana* tolerance to salt stress. Germination tryout results exposed greater salt tolerance at lower Fe treatments than at higher Fe treatments. Some indicators, such as iron leakage, levels of sodium, potassium and reactive oxygen species of roots also indicated increased tolerance to salinity in case of low Fe treatments. Reduced glutathione levels of plants were caused by salt stress and it designated further increased damage of an oxidation state. Nevertheless, high Fe content tended to cause more damages to the redox system than low Fe content. In conclusion, Fe content had a significant function in resistance to salinity by plants. The main explanation of the resistance to salt by plants may be the Fe's ability to bring about changes in the redox potential.

Key words: Iron content, Salt stress, Active oxygen, Redox system.

Introduction

Soil salinization is a key factor that restricts the productivity of agricultural crops. Some basic mineral nutrients are necessary for the growth of plants. However, soluble salt in the soil is harmful to many plants (Hasegawa *et al.*, 2005). High salinity may give rise to strong ionic and osmotic stress in glycophytes (non-halophytes) plants and it may even lead to their death (Hasegawa *et al*., 2005; Hossain & Dietz, 2016). The change of ionic and osmotic potential causes damage or growth reduction, resulting in the decrease or the collapse of the cell membrane; accumulation of active oxygen (ROS); toxic or hazardous substances; lower photosynthesis, metabolism disorders and malnutrition in crops (Flowers, 2004; Munns & Tester, 2008; Zhang *et al*., 2013). Under salt stress, the osmotic action decreased the seeds water absorption. Salt ions (Na^+, Cl^-, SO_4^{2-}, etc.) could be considered as toxic substances which may inhibit the germination of seeds (Gorai *et al.,* 2014). Thus, plant cell osmotic potential could be an adjustment tool and osmotic regulator through osmotic mechanisms, as well as improve salt tolerance of plant (Dubey & Singh, 1999). In the case of salt stress environment, the ion homeostasis in plant cells are ravaged and excessive ions in the cytoplasm, especially Na^+, may harm the metabolism of plant cells. On the basis of absorption and transport of ions, the plant roots have selective absorption capacity of Na^+ and K^+ from the soil, which were observed for the first time by Wang and Shu (1994). As it has been demonstrated by studies, the tolerance towards K^+ and Na^+ and their transport selective ability were significantly higher than that of salt in plants (Guo *et al*., 2015). The changes of K^+ content in some plants increased with the rise of Na^+ concentration to preserve the relative stability of the K^+/Na^+ ratio to further safeguard a good plant growth (Zhang *et al*., 2013).

The scavenging of reactive oxygen species in plants are dynamically balanced under normal physiological conditions, but the structure of these reactive oxygen (ROS) removal agents and cell membrane permeability were damaged by severe salt stress (Gao *et al.,* 2014; Jiang *et al*., 2016) and enhanced oxidative stress. Therefore, it is imperative to study higher plants salt tolerance and cellular antioxidant related genes. There are some methods to improve plants salt tolerance such as the exercises of salt resistance; growth regulators like auxin, cytokinin, ethylene and abscisic acid; using plant genetic engineering techniques namely transgenic technology cultivation of resistant to salt varieties, etc. It was reported that the polyploidic plant was anti-salt tolerance and could enhance the accumulation of potassium in plants (Chao *et al.,* 2013). In almost all organisms, Fe is found in many ubiquitous protein cofactors involved in key metabolic pathways, including the effect of low iron level on salt tolerence in *Arabidopsis thaliana* biosynthesis of chlorophyll, photosynthesis and respiration (Marschner & Marschner, 2012). The redox potential of Fe^{2+} /Fe^{3+} permits its use in many protein complexes in the form of the heme or iron sulfur clusters, especially those involved in electron transfer (Curie & Briat, 2003). The lack of Fe reveals its vital task, unambiguously in severe anemia of mammals and Fe chlorosis of plants (Briat *et al.,* 1995; Briat, 1999). Although Fe is an indispensable element in the cell, it can react with oxygen and as consequence, generates noxious ROS which harms the plant (Halliwell & Gutteridge, 1992). Therefore, appropriate Fe content is predominantly vital to sustain the normal metabolism and function in plants (Ferraro *et al.,* 2003).

The interaction between Fe and other elements may affect the growth of plants. It was reported that effects of iron toxicity were the direct factor of inhibition for roots length (Ward *et al.*, 2008). Iron nutrition can also affect the absorption of cadmium to regulate the metabolism of plants. Cumulative iron fertilizer can boost the competitive ability of iron in the interaction between Fe and cadmium; therefore, reducing the toxic effect of heavy metal cadmium (Gao *et al*., 2011). Salt stress induce plants to produce ROS, including hydrogen peroxide (H_2O_2), hydroxyl radical and superoxide ion (Hasegawa *et al.*, 2005). It can also lead to amplified lipoxygenase and NADPH oxidase activity in plants by attacking antioxidant enzymes and deteriorating their activity, causing membrane lipid peroxidation, membrane damage and eventually leading to oxidative stress and interference with the antioxidant system (Fadzilla *et al*., 1997). Iron is a kind of plant growth and development of the necessary trace elements, which produce interaction effects with salt stress. The use of iron chelates, such as Fe-EDDHA can improve salt stress tolerance of some crops (Nenova, 2008). Salt stress and iron deficiency stresses can make *Malus xiaojinensis* (iron salt high efficiency genotypes) to absorb more Fe^{2+} than *Malus baccata* (iron deficiency in sensitive genotypes) which could also maximally decrease the negative impact of salt stress and augment the tolerance of plants to salt stress (Siham *et al.*, 2015).

As a crucial trace element, Fe is involved in cell detoxification, plasma membrane redox systems and other processes; therefore, we infer that change in the Fe content may have certain purposes in plant responses to salt stress. In the present study, we used gradient Fe levels to treat *Arabidopsis thaliana* plants to demonstrate the affiliation between changes in Fe content and plant responses to salt stress based to two hypotheses: Fe plays a vital role in the oxidation-reduction reaction (1), low iron level improves salt tolerance by changing the redox system (2). We then discussed the outcomes to enlighten the required tasks of iron in plant response to salt stress.

Materials and Methods

Plant material and growth conditions: The seeds of *A. thaliana* Col0 wild type were washed and vernalized. Seedlings of uniform size were chosen from treatment medium at 8d after sowing. The treatment media had low, normal and high Fe contents (50μM, 100μM and 150 μM EDTA-NaFe, respectively) and it also contained 150 mM NaCl in all three mediae. The low, normal and high Fe contents with no added NaCl were the controls. The range of Fe contents was chosen as it did not greatly affect *Arabidopsis* growth in preliminary experiments using 5, 50, 100, 150 and 200 μM (data not shown).

Ion content measurement: *Arabidopsis thaliana* plants were washed using deionized water and they were wiped dry, then they were treated for 15 min in 105°C and oven-dried at 65°C. Dried *Arabidopsis thaliana* was ground in a pestle and 0.1 g of the powder was added to 5 ml of HNO_3 and left to stand for 30 min. The samples were then placed in a microwave digestion system for 25 min at 180°C with a constant volume of 25 ml. Ion contents were then determined by ion spectrum analyzer. Each treatment measurement was replicated thrice.

Measurements of net Na^+ fluxes with NMT: Net fluxes of Na^+ were measured using Non-invasive Micro-Test Technology (NMT100 Series, Xuyue (Beijing) Sci. & Tech. Co., Ltd. Beijing, China). Na^+ ion-selective microelectrodes with root hair area were manufactured. The samples were measured in the testing solution at pH 6.0 (0.1 mM $MgCl_2$, 0.1 mM KCl, 0.1 mM $CaCl_2$, 0.2 mM Na_2SO_4, 0.3 mM MES, 0.5 mM NaCl, pH 6.0, adjusted with choline and HCl). Only electrodes with a Nernstian slope >50 mV/decade were used in this study. Na^+ fluxes were calculated by using the JCal V3.3 (a free MS Excel spreadsheet, youngerusa.com or xuyue.net). Fick's law of diffusion ion flux formula was used:

$$J = -D0 \cdot (dc/dx)$$

where J represents the ion flux, dc is the ion concentration gradient, dx is the distance between the two points, and D0 is its diffusion constant. The direction of the flux is derived from Fick's law of diffusion.

Ion leakage assay: The electrolyte leakage test was performed as described previously (Lee *et al*., 2002). The leaves of treatments and controls were pumping for 30 min with a vacuum pump before adding 10 mL of distilled water. Then, they were placed on a shaker and shaken for 1 h at room temperature. Finally, each glass sample tube was placed in a boiling water bath for 15 min. After autoclaving, the percentage of electrolyte leakage was calculated as the ratio of the percentage of electrical conductivity.

Measurement of ROS levels: Intracellular levels of ROS were measured with DCFH-DA (2,7-dichlorodihydro-fluorescein diacetate; Sigma, USA). DCFH-DA was used at a final concentration of 20μg/mL with roots incubated with DCFH-DA for 10 min in darkness at room temperature; afterwards, they were washed three times with phosphate buffer solution (PBS). Fluorescence intensity was measured using confocal microscopy with excitation at 488 nm and emission at 525 nm.

Glutathione measurement: The contents of reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined with a GSH and GSSG Assay Kit (Beyotime, PR China). Colorimetric determination was conducted using a Biotek Synergy microplate reader (Biotek, USA).

Results

Germination of *Arabidopsis* seeds and salt tolerance of seedlings at different Fe treatments: High salinity causes strong ion and hypertonic stresses in plants. Ion and hypertonic stresses could lead to oxidative stress which could inhibit seedling growth. Compared to low Fe (50μM), the root growth for higher Fe treatments was significantly repressed at 150μM salt stress and the number of lateral roots was also reduced. Leaves under higher Fe (150μM) conditions also showed bleaching, but leaves at lower Fe (50μM) were still green (Fig. 1A). There were no significant differences in Arabidopsis under different Fe treatments without salt stress.

Seeds germination rate is an indicator which reflects the effects of plant growth. The Fe content alone had little effect on seeds germination; nonetheless, under salt stress, different Fe contents led to various germination and growth phenotypes. Salt stress had a strong impact both on the seed germination and the growth of the plant (Fig. 1B). Yet, seeds germination rate was higher under Fe lower treatments (50μM) than higher Fe treatments (150μM). In the environment of salt stress, many seedlings in higher Fe treatments (150μM) died after germination, but seedlings at low Fe (50μM) continued to grow.

The level of potassium and sodium in plant under salt stress: The Na^+ content in controls without salt stress showed no significant difference (Fig. 2A). Salt stress could greatly increase the Na+ content level, but it was much less in lower Fe (50μM) and nearly the half of that in higher Fe treatments. The Na+ content in higher Fe treatments (150μM) was slightly greater than that without salinity illustrating that plants in higher Fe (150μM) were more susceptible to salt stress. The K^+ content of plants decreased rapidly in the situation of salt treatment (Fig. 2A) affecting the growth of Arabidopsis. This displayed that salt stress affected plant growth. The K^+ content exposed only small changes without salt treatment; however, that content was much smaller in lower Fe treatments and under salt stress. This indicated that lower Fe could enhance salt tolerance compared to higher Fe treatments.

By measuring real-time kinetics of Na^+ flux without salt treatment, we found that the plant root hairs exhibited Na^+ efflux rates mainly, but when those plants were treated by NaCl, they presented Na^+ influx (Fig. 2B). This result suggested that salt stress enhanced Na^+ absorption of plant. The mean rate of Na^+ flux with an elevated Fe treatment (150μM) exposed significantly higher Na^+ influx rates than lower Fe treatment (50μM) (Fig. 2C), hence the accumulation of sodium ions in the plant. It showed also that low Fe treatment can decrease the seedling root hair zone of sodium ion absorption under salt stress, thus slowing down the imbalance of K^+/Na^+ ratio in the cytoplasm to uphold normal cell function in plants.

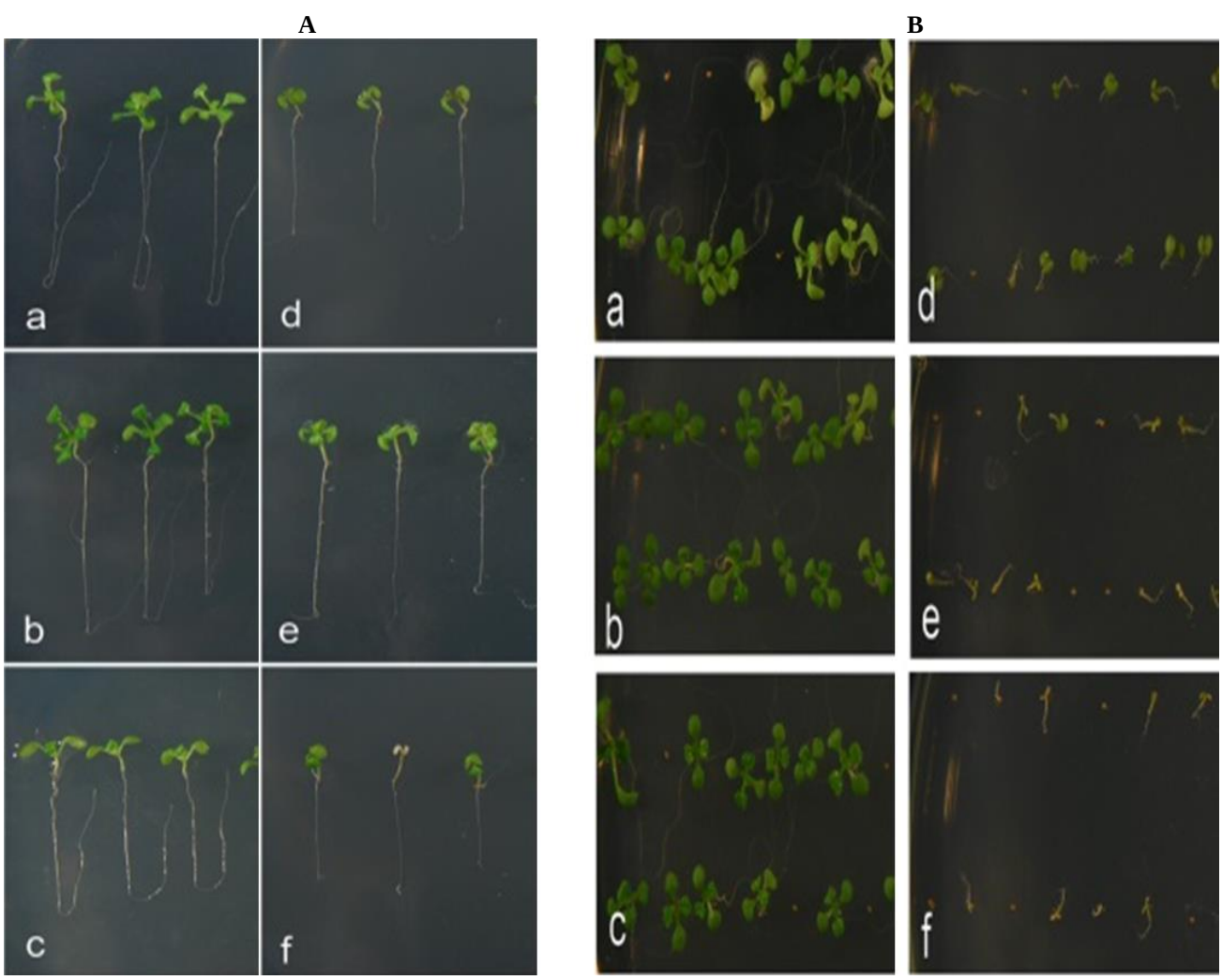

Fig. 1. The phenotype of different Fe treatments under salt stress:
(A) The state of seedling of WT at different Fe treatments under salt stress. (B) The germination and growing of WT at different Fe treatments under salt stress. (a) Fe 50 μM, (b) Fe 100 μM (control), (c) Fe 150 μM, (d) Na 150 mM, Fe 50 μM, (e) Na 150 mM, Fe100 μM and (f) Na 150 mM, Fe 150 μM.

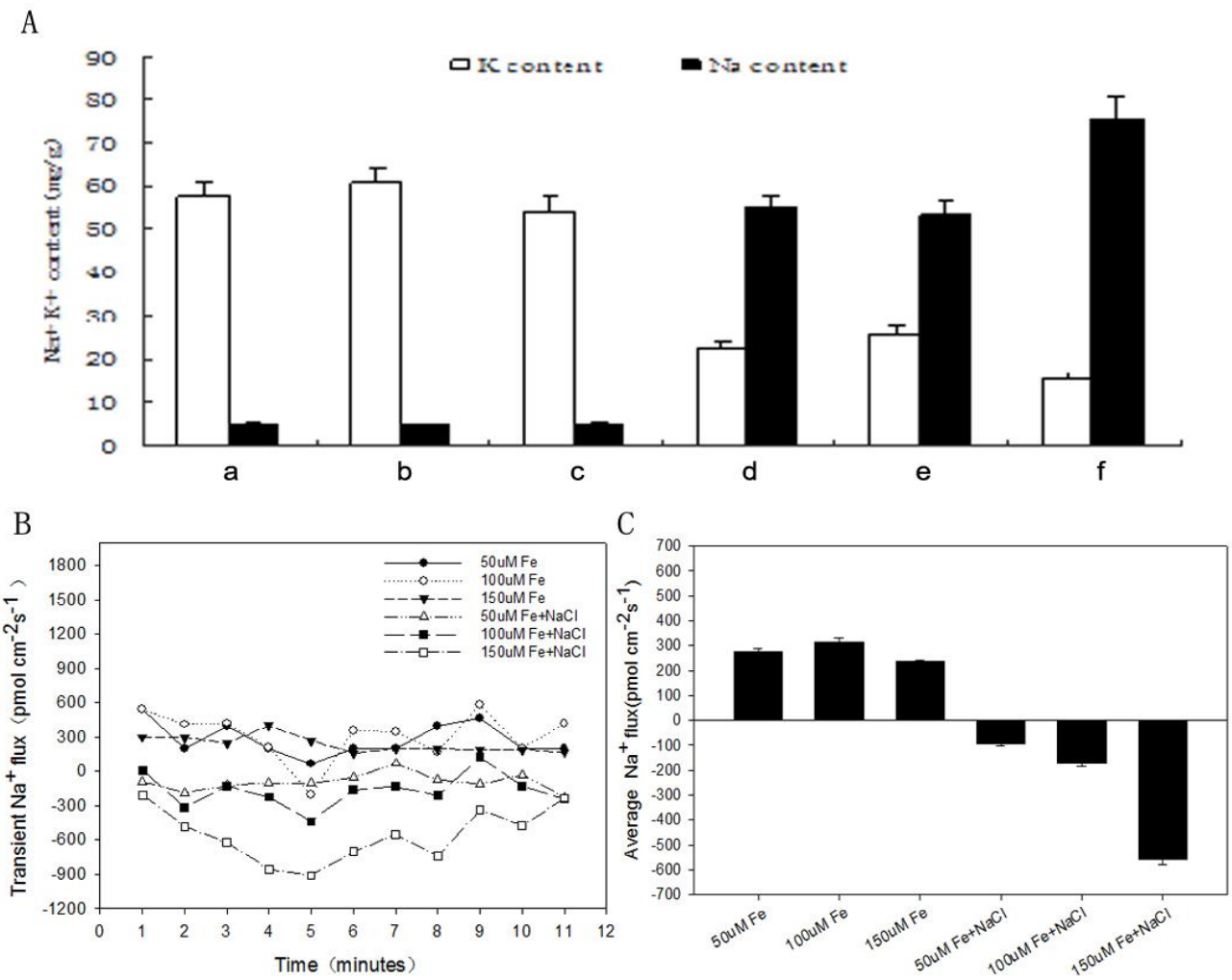

Fig. 2. The Na^+ and K^+ level of different Fe treatments under salt stress.
(A) The Na^+ and K^+ levels at different Fe treatments under salt stress. (B) The transient Na^+ flux of plant root hair zone at different Fe treatments under salt stress. (C) The average Na^+ flux of plant root hair zone at different Fe treatments under salt stress. (a) Fe 50 μM, (b) Fe 100 μM (control), (c) Fe 150 μM, (d) Na 150 mM, Fe 50 μM, (e) Na 150 mM, Fe100 μM and (f) Na 150 mM, Fe 150 μM. Data were the mean ± SEM of at least three different experiments.

Changes of ion leakage rate of plant under salt tress: The ion leakage rate of cell membranes is often used as an indicator to reflect salt sensitivity. We compared the ion leakage changes for different Fe treatments with or without salt treatment over several days. The ion leakage rate under normal conditions was very low, indicating a good plant growth and undamaged cell membranes. Ion leakage rate rose as salt treatment time increased (Fig. 3) and was much higher than in controlled plants. Ion leakage rate for higher Fe treatments was > 60% after 6d under salt stress and > 80% after 9d, indicating that cell membranes had been severely damaged and ions were leaking out.

ROS content and redox potential changes in plants under salt stress: H_2O_2 is an important ROS and its content can indicate different degrees of salt tolerance for the various Fe treatments. The fluorescence intensity represents the content of H_2O_2 and that fluorescence was greater than without salt treatment; however, the fluorescence zone also differed with the Fe treatments (Fig. 4A). Only root tips fluoresced for the lower Fe treatments while almost all root zones fluoresced for higher Fe treatments, indicating extreme H_2O_2 content in higher than in lower Fe treatments. This was also validated by quantification of fluorescence intensity (Fig. 4B).

Glutathione has two forms: oxidized (GSSG) and reduced (GSH). GSH and GSSG can control the thiol–disulfide to coordinate redox potential. The redox potential balance is damaged by the rapid accumulation of ROS; therefore, the GSH/GSSG ratio is an indicator of altered redox potential. GSH/GSSG contents differed for different Fe treatments (Fig. 4C). The GSH contents were toweling in lower Fe treatments than in normal and higher Fe treatments regardless of with or without salt treatment, suggesting that lower Fe could induce lower redox potential and increase tolerance to salt stress. In contrast, higher Fe induced an elevated redox potential and increased susceptibility to salt stress damage.

When plants are faced to salt stress and low iron, it leads to a low redox potential and low ROS levels which bring to a higher salt tolerance, while the salt stress combined to a higher iron level convey to the increase of the redox potential and ROS levels, those ROS will damage the mitochondria which will cause the plants death, and the result will be a lower salt tolerance (Fig. 5).

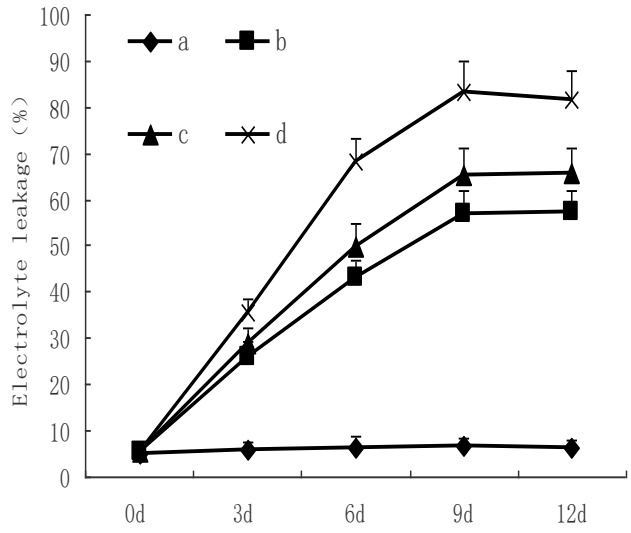

Fig. 3. The ion leakage of different Fe treatments time under salt stress.
(a) Fe 100 µM (control), (b) Na 150 mM, Fe 50 µM (c) Na 150 mM, Fe100 µM and (d) Na 150 mM, Fe 150 µM. Data were the mean ± SEM of at least three different experiments.

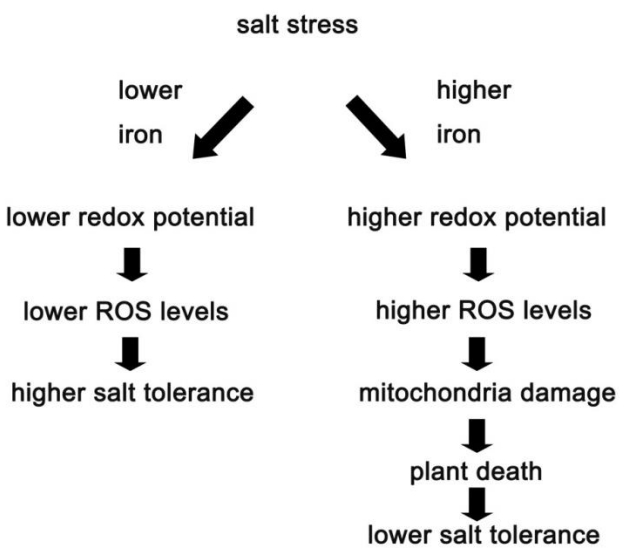

Fig. 5. The possible mechanism of salt stress for different Fe treatments.

Discussion

There was no significant difference in the short-term performance of *Arabidopsis thaliana* under the treatment between low Fe content (50µM) and normal Fe content (100µM). However, the effect of high Fe content (150µM) on the plants was much more noticeable, especially in the treatments of salt stress. Salt stress, which could cause the ion and osmotic stresses in plants, may lead to secondary stresses such as lack of nutrition, ion toxicity, low photosynthesis and oxidative stress (Mittler, 2006; Riveraingraham *et al.*, 2016). This investigation found that the sodium content in the cell membrane increased rapidly and led to competition effect with potassium ion under salt stress. High salinity leads to ion stress which is mainly caused by Na^+ ion poisoning. Those Na^+ ions are toxic to many plants, primarily due to their replacing of potassium (K^+) ions in biochemical reactions (Zhu, 2001; Zhu, 2002). The Na^+ content will rise rapidly under high salinity stress and thereby the growth of plants is repressed. K^+ ions that were involved in cell arduousness, membrane potential, activity of many enzymes, protein synthesis and conformation play critical functions in plants. They can activate a variety of enzymes and also function as a cofactor. Na^+ and K^+ ions have similar biochemical properties; thus, high concentrations of Na^+ ions have a strong inhibitory effect on the absorption of K^+ ions in roots (Tester & Davenport, 2003). Plant cells need to preserve adequate potassium nutrition and appropriate proportion of K^+/Na^+ in the cytoplasm in order to avoid cell damage and malnutrition (Serrano *et al.*, 1999). The results displayed that Fe plays an important role in balancing the ions effects in plant cells and the high Fe concentration (150µM) supply was unfavorable for plants to maintain the proportion of K^+/Na^+. Besides for, compared to the treatments of control (100µM Fe) and low iron (50µM Fe), Na^+ content was significantly elevated under high iron (150µM Fe) which caused a slower growth of plants. The reason of the damage from high iron content might be the toweling concentration of Fe which might lead to the disorders of ions absorption in the cytoplasm of the cell.

In case of salt stress, the photosynthetic capacity was reduced and the excess light energy could be used for the excitation and production of reactive oxygen species (ROS). Excessive accumulation of reactive oxygen species will lead to membrane lipid peroxidation which is harmful to plant cells (Huang & Guo, 2005). It is imperative for plants to remove ROS rapidly. Under normal growing conditions, plants rely on free radical scavenging system of chloroplast and mitochondria which could maintain the low level of free radicals in cell and normal physiological metabolism (Asada, 2006; Navrot *et al.*, 2007; Win & Ookawa, 2016). Glutathione is a non-protein thiol compound with many physiological purposes in plants such as the regulation of plant cell death (Henmi *et al.*, 2001), the adaptation of oxidative stress (May *et al*., 1998) and the presence of cysteine that confers its biological properties mainly as an antioxidant key through its involvement in cell redox homeostasis (Dubreuil-Maurizi & Poinssot, 2012; Yamamoto *et al.*, 2016). As a strong reducing agent, GSH was one of the most effective active oxygen scavengers when plants were subjected to oxidative stress. The concentration and distribution of glutathione as well as the ratio of GSH/GSSG were closely related to the aptitude of plants to resist to oxidative stress (Vlachaki & Meyn, 1998). It was reported that the loss of GSH synthetic gene could change the glutathione content, thus, decreasing the antioxidant capacity and magnifying the oxidative damage in the transgenic plants of orange (Creissen *et al.*, 1999).

In this study, active oxygen content under low Fe and high Fe treatment increased in different degrees. Data portrayed that the high concentration of iron could increase the content of hydrogen peroxide in plantlow

irons significantly when they were planted in salt stress situations. It was also observed the upsurge of electrolyte permeability under the high iron content, indicating that the plants suffered from more severe oxidative damage. There was no significant difference of active oxygen content between low Fe (50 μM) and control (100μM Fe) treatments, but electrolyte permeability was lower and the oxidation potential was toward the prototype GSH direction that had a more powerful scavenging active oxygen trend and showed higher salt tolerance.

It is not feasible to raise the active oxygen scavenging mechanism only by increasing the ferrite concentration, but the study disclosed that the suitable low Fe treatment could lead to a better growth state which is involved in seedling growth, seed germination, the content of sodium and potassium ions, the ion leakage and the reactive oxygen accumulation. Fe plays a crucial role on the oxidation-reduction reaction. The data of GSH/GSSG confirmed the change of redox state under different iron regimes. It is possible that Fe content impacted the redox system by changing the redox potential. The redox reaction is related to many abiotic stresses; thus, iron content may not only regulate the salt stress, but it may also be involved in other abiotic stresses such as drought and cold stresses. Those hypotheses should be confirmed by further researches.

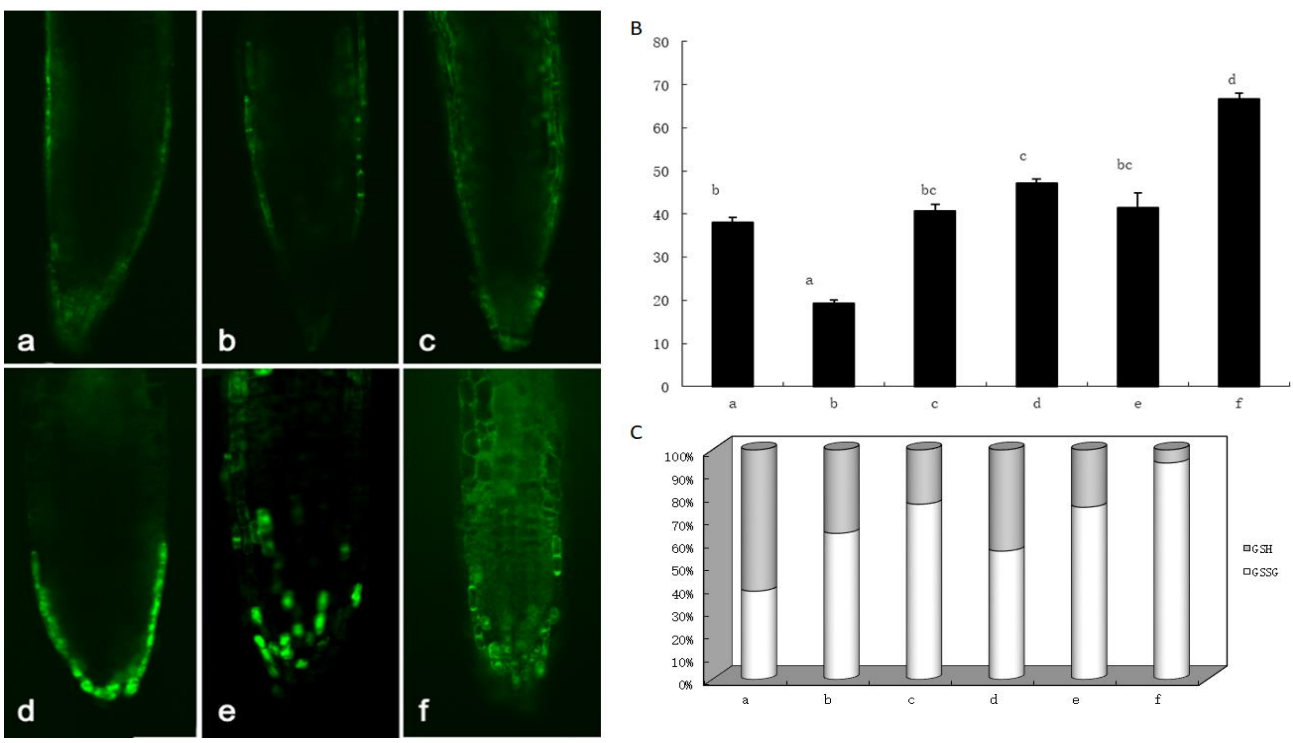

Fig. 4. ROS content for different Fe treatments under salt stress
(A) The fluorescence intensity of H_2O_2 for different Fe treatments. (B) The quantification of fluorescence intensity of H_2O_2 for different Fe treatments. (C) The GSH/GSSG ratio of different Fe treatments under salt stress: (a) Fe 50 μM, (b) Fe 100 μM (control), (c) Fe 150 μM, (d) Na 150 mM, Fe 50 μM, (e) Na 150 mM, Fe100 μM and (f) Na 150 mM, Fe 150 μM. Data are the mean ± SEM of at least three different experiments. Different letters represent statistically different means at $p<0.05$ (one-way ANOVA analysis with Duncan post-hoc test)

Acknowledgements

Authors are thankful to the Modern Agricultural Industry Technology System (CARS-27); to the Special Fund for Agro-scientific Research in the Public Interest (201303093); to the National key technology R&D program (2014BAD16B03) and to the Collaborative Innovation Center for Eco-environmental Improvement with Forestry and Fruit Trees (CEFF_2018_014207_000024).

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(Received for publication 28 January 2018)