

RICE ADAPTS TO ALKALI STRESS BY MICROSTRUCTURAL CHANGES

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Abstract

Response of four rice varieties namely Suijing 5, Longjing 27, Fujisawa 138, and Koshihikari to salt-alkali stress can assist in understanding the physiological and molecular mechanisms of salt-alkali stress. The sensitivity differences of microstructural changes under alkali stress between different rice varieties were examined and found that the alignment and integrity of root cells of different varieties of rice were compromised by alkali stress. The root cells of Longjing 27 were more sensitive to alkali stress, and its morphology more easily changed. In different rice varieties the formation of branch roots, the proportion of vascular columns and large vascular bundles in the root cross-section were increased along with the increase of the diameter of the vascular column and large vascular bundles, and maintained consistent transport of nutrients to compensate for the root function. In all resistance to alkali stress was chloroplasts and mitochondria were also affected by alkali stress, while Suijing 5 was more resistant to alkali stress. Chloroplast structure and function in Suijing 5 were relatively intact. Suijing 5 could still complete photosynthesis but could not complete the transport of synthetic products. The structure and function of mitochondria and chloroplasts in the other three rice species were severely impaired, and photosynthesis and product transportation were blocked. Suijing 5 was the best alkali resistant rice variety according to microstructural analysis.

Key words: Alkaline stress; Rice variety; Cell microstructure; Response analysis.

Introduction

Different plant species have different ways of adapting to stress. These different mediation mechanisms can give certain plants a competitive advantage over other plants (Mukherjee *et al.*, 2021). Even if the difference in the mediation mechanism is small, it may cause plants to adapt in ways that lead to the evolution of new species (Lei *et al.*, 2006). Natural stressors include biological factors (pests, diseases, etc.) and non-biological abiotic environment (Geographical conditions, soil types, extreme climate, etc.) (Kosová *et al.*, 2011). Salinized soil is considered an abiotic stressor of plant growth. Species, breeds, and gene families (Xiong *et al.*, 2002) exhibit adaptive differences when exposed to this abiotic stressor (Negrão *et al.*, 2013; Yamane *et al.*, 2008). These adaptive differences are derived from genes and proteomes (Barkla *et al.*, 2013), which are reflected in changes to plant growth and microstructure (Miyake *et al.*, 2002; Zhao *et al.*, 2014). Understanding the response of plants to salt-alkali stress is important to understand the salt and alkali resistance of plants. Research on the response of plant microstructure to saline-alkali stress has been performed on a large number of plant species. Relevant research shows that plant chloroplast is the most sensitive subcellular structure to salt stress (Zhu *et al.*, 2000; Jiang *et al.*, 2001) which can cause membrane system disorder disintegration (Chinnusamy *et al.*, 2005). Under these conditions, the blade PSII reaction center could also be destroyed (Geissler *et al.*, 2009). Under high-intensity stress, the chloroplast will rapidly age, the lamellae structure will become distorted, the matrix will become thinner, the osmiophilic granules will be hollowed out, and the structure and quantity of

mitochondria will change accordingly. This can even trigger programmed cell death (Petrov *et al.*, 2015; Qing *et al.*, 1999).

Rice (*Oryza sativa* L.) is one of the most widely distributed and commonly cultivated plant species by human beings. More than half of the world's population considers rice a staple food, especially in developing countries. It is one of the most important food crops in the world, having also been planted near N50° in the northern latitudes of China. This area is covered in rich soil, much of which has a concentration of saline-alkali. Rice has an adaptability to the saline-alkali environment, and its yield and quality are affected by saline-alkali stress. However, there are still some differences between varieties. Studies on salt-alkali stress in rice have focused on morphological and physiological functions (Flowers *et al.*, 1995). Some microscopic studies have also shown that there are differences in the ultra-structure of leaf cells across different varieties (Li *et al.*, 2000). There are no relevant studies about whether this difference is related to the ability to adapt to the saline-alkali environment. In this study, we treated the seedlings of different rice cultivars with saline-alkali stress and analyzed the response of root morphological changes, leaf cell and subcellular structure of the different cultivars. We then examined the microscopic characterization and changes in the operational mechanisms of each organelle function of rice under alkali stress from a microscopic perspective.

Materials and Methods

Site and soil details: An experimental field was established in Jiamusi City, located in the east part of Heilongjiang Province, China. This region has a climate

of cold humid monsoon with annual average temperature of 3°C, annual average precipitation of 468.4 mm, annual sunshine of 2046.2 h, and annual active accumulative temperature of 2500°C. The annual average frost-free period is 128.9 days. The organic matter of meadow black soil was 2.49%, available nitrogen was 86.3 mg·kg⁻¹, available phosphorus was 64.6 mg·kg⁻¹, available potassium was 79.9 mg·kg⁻¹, total nitrogen was 0.14%, total phosphorus was 0.14%, total potassium was 3.12%, pH was 6.5. The soil Pb background value was 0.24 mg·kg⁻¹, Cd was 0.031 mg·kg⁻¹.

Test materials: The tested rice cultivars included four cultivars: Longjing 27, Suijing 5, Fujisawa 138 and Koshihikari. Rice Research Institute of Heilongjiang Academy of Agricultural Sciences of China provided Longjing 27 and Suijing 5 samples. Waseda University of Japan provided Fujisawa 138 and Koshihikari samples.

Experimental design

Early culture of seedlings: The seeds were immersed and sterilized by 0.1% potassium permanganate for 24 hours, then spread in a tray containing quartz sand. The seeding amount was 100g/m². The tray was covered with 2cm of sand then the seeds were spread evenly, 0.5cm of sand was then poured on top of the seeds bringing total sand height to 2.5cm. The incubator was set at a temperature of 30°C, and after 72 hours the seedlings were removed from the incubator. After removal, the seeds were grown at room temperature to the two leaf stage.

Late culture of seedlings: The culture solution was carried out in the Kimura culture medium. The upper part of the culture box was 30×20cm. The side and bottom were closed with a light-shielding paper. The culture box was covered with a 1cm thick foam carrier. The carrier plate was evenly penetrated with 20 culture holes with a diameter of 1.0cm (adjacent whole margin 5 × 5cm). A two leaf seedling was selected from each well. The main stem was wrapped with a thin sponge strip, inserted into the culture well, fixed on the culture plate, and cultured in the wood culture medium. The culture solution was replaced every 2 days.

Treatment of reagent and period: The treatment reagent was a mixed alkali solution of Na₂CO₃ and NaHCO₃. The mass ratio of these two kinds of reagent was 1:3, and the mass concentration of the aqueous solution was 0.20%. Control (CK) and 0.20% treatment (CL) were conducted under outdoor conditions with 3 replicates. When the 3rd-instar seedlings were completely unfolded, the culture liquid was replaced with the Kimura culture liquid (now referred to as the treatment liquid) containing the mixed alkali. The seedlings of CK were grown in the Kimura culture liquid, and the treatment and culture liquid was replaced every 2 days. After 10 days, the middle section of the first fully expanded blade was removed for observation and measurement.

Biological indicator measurements: Measurement of vascular tube diameter, center column diameter, root diameter: We took 20 roots from the control and treatment group and observed the cross section under an electron microscope. Data was obtained by the electron microscope image processing software. Multiplying the ratio of each index by 100% gave us the ratio of the vascular beam diameter to the medium cylinder diameter ratio, the center pillar diameter/root diameter, and the vascular bundle diameter/root diameter.

Measurement of cell length and width: We took 20 roots from the control and treatment group and observed the longitudinal section under an electron microscope. Data was obtained by the electron microscope image processing software. The longitudinal direction is the cell length and the lateral direction is the width. The ratio of each index was obtained from the cell aspect ratio, the mid-column cell aspect ratio, the stele cell length/thin wall cell length, and the stele cell width/thin wall cell width.

Measurement of the number of vascular bundles: We took 20 roots from the control and treatment group and observed and counted the number of vascular bundles under an electron microscope. Data was obtained by the electron microscope image processing software. The number of vascular bundles was obtained by taking the average value of the bundles.

Measurement of vascular bundle area, stele area, root cross-sectional area: We printed the image to be tested on standard paper, cut the indicator image to be measured, and then weighed it. The standard length was the side length, which was obtained by using the software. We drew a square on the same standard paper, calculated the area and weighed it. The relevant data was obtained by the calculation of the specific gravity method. Multiplying the ratio of each indicator by 100% gave us the ratio of vascular bundle area/mid column area, stele area/root cross-sectional area, vascular bundle area/root cross-sectional area.

Observation objectives and treatment methods

Root cell observation method: We used a German Leica DM4000B electron microscope to observe cell alignment in the cross-section and longitudinal section of each part of the crown. After the sections were stained with a methyl red solution, photographs were immediately taken at an eyepiece magnification of 10 × 122 and an objective magnification of ×10 times.

Processing method of blade ultrastructure: Procedures of the leaf treatment: the removed leaf samples were immediately placed in 2.5% glutaraldehyde for pre-fixation. We then vacuum pumped to sink the material, and placed it in a refrigerator at 4°C for 2 days. The materials were then rinsed three times for 10 minutes each with pH 7.2

phosphate buffer, fixed with 2% tetrahydrogen monoxide for 1.5 h, and again rinsed three times (for 10 minutes each) with pH 7.2 phosphate buffer. Dehydration was carried out with 50%, 70%, 80%, 90%, and 100% ethanol for 10 to 15 minutes each time. Dehydration with 100% ethanol was performed three times and other dehydration solutions one time, then replaced the solutions with 100% ethanol: acetone = 1:1, pure acetone, each for 10 minutes. Soaking the samples in pure acetone: epoxy resin 812 embedding agent (1:1, 1:2, 1:3) for 1h, 2h, 2-3 days respectively. The samples were placed in incubator at a constant temperature for polymerization, setting conditions at 40°C for 17h, at 45°C for 24h, and at 60°C for 24h. An ULTRATUTE ultra-thin slicer was used to slice the samples. The slices were stained with uranyl acetate and lead citrate at 25°C for 15-20 minutes, rinsed with double distilled water and placed in a culture dish. The photographs were observed under a JSM25610LV transmission electron microscope.

Data processing: The data was analyzed using Excel, DPS and SPSS software.

Results and analysis

Effect of alkali stress on rice root cells: Since the root is the first organ to be exposed to the alkali solution, rice will first respond to the stress at its root. Therefore, the morphology of rice root cells was first observed. Our results showed that the trend of morphological changes in root cells were the same for the four rice cultivars. Further observation indicated that there were differences in the sensitivity of rice varieties to alkali stress. Longjing 27 and Koshihikari were examples of the differences of sensitivity in this section.

Observation of cross-section cells of rice roots: Through observing the cross section of the root cells via electron microscope (Fig. 1), we found that the cells in the radicles of the control group were arranged neatly, clearly, compactly, with regular cell shape and stereoscopic properties. This indicated that the root cells had biological activity (Fig. 1-a). The epidermis of the control group was covered with dense root hair tissue (Fig. 1-aH), and cortical parenchyma cells were arranged from the inside to the outside in a small to large radial pattern. After 10 days of treatment with 0.2% alkali solution, the cortical parenchyma cells were loose and disordered compared with the control group, the color distribution after staining was uneven, and the root hair of the epidermis was short and bud-like (Fig. 1-b-5). After treatment, the number of primary large vascular bundles in the root vascular column was decreased by 1-3x compared with the control, with large vascular bundles that were not fully developed (Fig. 1-b-9-m). In the control group, the endothelial cells and the stele sheath cells were closely arranged and the cell size remained the same. The endothelial cells and the stele sheath cells in the treatment group were loosely arranged, and the ridge area was obvious and lignified. The number of branches in the control group was small but increased after treatment.

The above results indicated that alkali stress significantly disrupted the neat and compact arrangement of rice root cells, inhibited the formation of large vascular bundles, and increased the number of undeveloped vascular bundles. Alkali stress inhibited the growth of root hair, promoted the lignification of roots and promoted the growth of shoots. There were some unclear brown areas in the cross section of the main root of the rice under alkali stress (Fig. 1-b-8-n). It is likely that the cell fluid leaked due to the high external osmotic pressure, and the degree of color change was related to the cell fluid exuded in this area, indicating that the physiological cell function of these areas had changed.

The diameter and area of the vascular bundle, stele, and root were all measured after alkali treatment. The results showed that the root trend of Longjing 27 was in accordance with Koshihikari. That is, the number and the diameter of vascular bundles, and the diameter of the stele of rice roots were decreased significantly, and the ratio of diameter to root diameter of the stele and the ratio of vascular bundle diameter to root diameter increased significantly (Table 1-1). The area of the vascular bundle in the stele of roots was significantly reduced due to alkali stress (Table 1-2), the proportion of the stele and vascular bundles in the cross section of roots increased significantly (Table 1-2). Although the number of vascular bundles, the ratio of its diameter, and the cross-sectional area to stele decreased after treatment, the ratio of its diameter and cross-sectional area to root was increased significantly showing that the single vascular bundle thickened after treatment, and the total cross-sectional area of intracellular vascular bundle was relatively increased. In order to adapt to the alkali environment, rice could maintain the transportation of nutrients and water by increasing the proportion of the vascular column and the vascular bundle in the root cross section to maintain growth and development.

Cells in a longitudinal section of rice: The longitudinal section of the root (Fig. 2) showed that the root cells of the control group were arranged neatly and orderly. The cortical parenchyma cells were stereoscopic, indicating that the root cells of the control group were active. After alkali stress treatment, the cells in each root were shorter and less stereoscopic, the parenchyma cells in the middle were polygonal, the parenchyma cells near the outer cortex were small cuboids, and the longitudinal length of some cells was shorter than its width. The parenchyma cells near the endothelium were round or short columnar (Fig. 2-b-4, 7), with relatively poor ordering, and the epidermal cells were detached (Fig. 2-b-4). The sheath cells were significantly shorter and thinner than that of the control group. In contrast to the results of cross-sectional observations, the longitudinal section cells were arranged more tightly than the cross-sectional cells. After treatment, there were numerous dark spots in the rice cortex parenchyma cells and the stele sheath cells (Fig. 2-b-7), and the color near the endothelial parenchyma cells was deeper, indicating that the cells in these sites were severely affected by alkali stress.

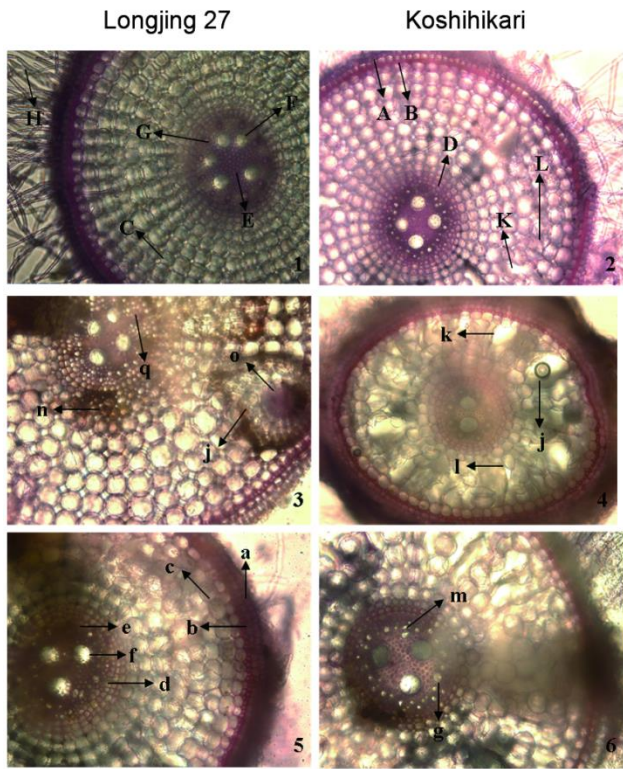


Fig. 1. Cell structure of radicle cross section.

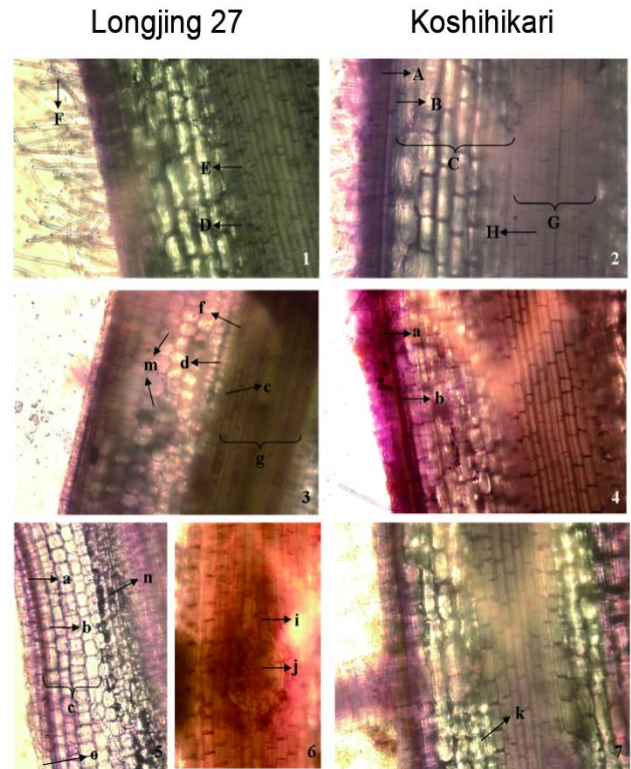


Fig. 2. Cell structure of radicle cross section.

Table 1-1. Diameter changes of vascular bundles, stele, and roots after alkali treatment.

Testing materials	Number of vascular bundles		Vascular beam diameter / medium diameter ratio (%)		Center column diameter / root diameter (%)		Vascular diameter / root diameter (%)	
	CK	CL	CK	CL	CK	CL	CK	CL
Longjing 27	5.1	4.3**	15.6	13.2**	22.7	30.7**	3.6	4.1*
Koshihikari	4.7	3.4**	18.2	13.7**	20.5	33.8**	3.7	4.7*

Note: “*” and “**” represent significant and extremely significant difference from CK

Table 1-2. Comparison of the area of vascular bundle, stele, and roots after alkali treatment.

Testing materials	Vascular bundle area/ center column area (%)		Center column area/ root cross-sectional area (%)		Vascular bundle area / root cross-sectional area (%)	
	CK	CL	CK	CL	CK	CL
Longjing 27	12.0	6.8**	5.2	9.4**	0.65	0.69*
Koshihikari	15.3	5.7**	4.2	11.7**	0.63	0.72**

Note: “*” and “**” represent significant and extremely significant difference from CK

Table 2. Changes in root length and width after alkali treatment.

Testing materials	Cell aspect ratio		Stele cell aspect ratio		Stele cell length / thin wall cell length		Stele cell width / parenchyma cell width	
	CK	CL	CK	CL	CK	CL	CK	CL
Longjing 27	2.77	0.89**	5.30	0.90**	0.863	1.011**	0.451	1.006**
Koshihikari	2.97	1.44**	3.74	1.95**	0.880	0.857	0.699	0.634*

Note: “*” and “**” represent significant and extremely significant difference from CK

The above results indicated that alkali stress significantly inhibited the longitudinal elongation of rice root cells, resulting in the slow growth of roots. The three-dimensional sense of cells was decreased due to stress, suggesting that the roots had been lignified. Cortical parenchyma cells and some of the stele sheath cells were necrotic due to alkali stress. The parenchyma cells near the endothelium were more seriously impaired, and the physiological characteristics of some cell populations had changed. The epidermal cells of the treatment group shed earlier than the control group. Results from the longitudinal section observation of root

cells showed that the cross section of the branches were beginning to form, and the roots of the control group were few indicating that alkali stress promoted the formation of branch roots. While the main root was destroyed, more branch roots would form to maintain the transportation of nutrients and water to ensure that the plants could grow and develop.

The aspect ratio of the thin wall cells and stele cells of rice after treatment was decreased significantly (Table 2) showing that the cell length was significantly inhibited and that the length of Longjing 27 was more inhibited. The ratio of stele cell length to parenchyma

cell length indicated that there was a reverse trend between these two varieties: compared with the stele cells of Longjing 27, the alkali stress had a greater ability to inhibit the length of the parenchyma cell but the length of the thin-wall cells and the stele cells of Koshihikari was proportionally inhibited. The ratio of stele cell width to parenchyma width showed that alkali stress significantly inhibited the width of parenchyma cells. For both Longjing 27 and Koshihikari, it was significantly inhibited. The above analysis showed that alkali stress had a significant or extremely significant effect on the morphology of rice root cells, and the inhibitory effect on parenchyma cells was stronger than that of the stele cells. However, the strength of the inhibition varied among different rice varieties. This study found that the root cells of Longjing 27 were more sensitive to alkali stress and its morphology was easier to change. The results of this analysis demonstrated the difference in alkali tolerance between different rice varieties based on the morphology and quantity of root cells.

Changes in photosynthetic pigments after alkali stress

Changes in pigment: It is well known that chloroplasts are severely impaired under alkali stress. Therefore, in order to investigate the changes of chloroplast structure in different rice varieties after alkali stress, we first measured the content of photosynthetic pigments. The results showed that the content of chlorophyll A, B, and carotenoids significantly decreased after treatment with 0.20% alkali solution for 10 days and the descend rate between the varieties was significantly different. After the treatment with 0.20% alkali solution, the photosynthetic pigments of all the rice varieties decreased significantly. The descend rate of photosynthetic pigments of Longjing 27 and Fujisawa 138 was higher than Suijing 5 and Koshihikari. The value of chlorophyll A/B of Longjing 27 and Fujisawa 138 showed a significant downward trend, indicating that the inhibition of chlorophyll A was stronger than that of chlorophyll B. The ratio of chlorophyll A/B of Suijing 5 and Koshihikari did not decrease or decreased insignificantly, indicating that chlorophyll A and chlorophyll B exhibited isocratic inhibition.

The pigment content of all tested varieties decreased significantly, indicating that alkali stress had significant effects on the pigment content of rice leaves (Table 3). Chlorophyll A in Longjing 17 and Fujisawa 138 was most sensitive to alkali stress, followed by chlorophyll B and carotenoids. The sensitivity of nucleoside was the smallest; there was no difference in susceptibility to alkali stress between chlorophyll A and chlorophyll B in Suijing 5 and Koshihikari, and carotenoid was least sensitive to alkali stress. This analysis indicated that alkali stress could inhibit the synthesis of photosynthetic pigments or promote the degradation of photosynthetic pigments. It also demonstrated that different rice varieties had different sensitivities to alkali stress.

Changes of the ratio of surface area of chloroplasts, mitochondria, and amyloplasts in cells: The above results indicate that the synthesis or degradation pathway of photosynthetic pigments are affected by alkali stress, which may be caused by structural damage of chloroplasts or by structural damage of mitochondria. Therefore, the relative number of mitochondria and chloroplasts was observed and measured for further study. The surface of chloroplasts, mitochondria, and amyloplasts in cells differed depending on the rice varieties (Table 4). After alkali stress treatment, the surface area ratio of chloroplasts and mitochondria to leaf cells of Longjing 27, Suijing 5 and Koshihikari increased significantly, but was significantly reduced in Fujisawa 138. The surface area ratio of amyloplasts to chloroplasts in Longjing 27 and Fujisawa 138 was significantly decreased but increased significantly in Koshihikari, and displayed no significant change in Suijing 5. The surface area ratio of mitochondria to chloroplasts showed that the surface area of mitochondria in Longjing 27 and Koshihikari was significantly increased and significantly reduced in Fujisawa 138, and there was no significant change in Suijing 5. The surface area ratio of mitochondria to amyloplasts was significantly increased in Longjing 27 and Fujisawa 138, while there was no significant change in Suijing 5 and Koshihikari. These results showed that the relative quantity or volume of chloroplasts and mitochondria did undergo significant changes under alkali stress, resulting in corresponding damage to their functions. While the volume of chloroplasts and mitochondria was significantly changed, its structure could also be affected. Therefore, it is necessary to further study these functional changes by observing the ultrastructure of chloroplasts and mitochondria.

Effects of alkali stress on the ultrastructure of rice leaf cells

Ultrastructure of leaf cells in Longjing 27: The intracellular chloroplasts of the Longjing 27 control group were fusiform, with rich lamellar structure, uniform and wide distribution of granule thylakoids, more basal lamellar layers, and observable stroma-thylakoid (Fig. 3). The chloroplasts of Longjing 27 were inlaid with many amyloplasts composed of larger starch granules (Fig. 3-2), with a small number of mitochondria and nuclei (Fig. 3-1, 3), and less osmiophilic granules. After alkali treatment, there were still a large number of chloroplasts in the cells, but their distribution was disordered and stacked (Fig. 3-7). The chloroplast was inlaid with a small number of smaller starch granules. Most of the chloroplasts were swollen (Fig. 3-6) and the thylakoids were biased toward the chloroplast side. The granules of the chloroplasts were significantly thinner and more chaotic (Fig. 3-4, 8), indicating that the thylakoids were degrading. Compared with the control group, mitochondria in the treatment group were still distributed around the chloroplast and relatively abundant. However, it was obviously swollen, its inner ridge was sparse, and the osmiophilic granules increased slightly, indicating it had suffered damage.

Table 3. Effect on photosynthetic pigments of testing materials under 0.20% concentration mixed alkali stress (Na₂CO₃+NaHCO₃ mg/g).

Testing materials	Chlorophyll A			Chlorophyll B descend rate (%)			Carotinoid descend rate (%)			Chlorophyll A/B descend rate (%)		
	CK	CL	DR	CK	CL	DR	CK	CL	DR			
Longjing 27	2.23	1.17**	47.4	0.66	0.38**	42.7	0.42	0.28**	32.2	3.36	3.08*	8.3
Suijing 5	2.24	1.54**	31.3	0.65	0.44**	31.5	0.46	0.36**	21.1	3.47	3.47	0
Fujisawa 138	2.52	1.51**	40.0	0.71	0.46**	34.6	0.49	0.34**	30.5	3.57	3.27*	8.2
Koshihikari	2.77	2.08**	25.0	0.82	0.62**	24.2	0.53	0.42**	20.1	3.37	3.34	1.1

Note: “*” and “**” represent significant and extremely significant difference from CK; DR means Descend rate (%)

Table 4. Changes in the surface area ration of chloroplasts, mitochondria, and amyloplasts in leaf cells.

Indicators	Longjing 27		Suijing 5		Fujisawa 138		Koshihikara	
	CK	CL	CK	CL	CK	CL	CK	CL
Chloroplasts / Cell	0.435	0.456*	0.494	0.688**	0.620	0.510**	0.590	0.681**
Mitochondria / Cell	0.017	0.031**	0.026	0.037**	0.041	0.028**	0.013	0.030**
Amyloplasts / Chloroplast	0.347	0.014**	0.116	0.115	0.123	0.017**	0.260	0.514**
Mitochondria / Chloroplasts	0.040	0.068**	0.052	0.053	0.066	0.055*	0.023	0.044**
Mitochondria / Amyloplasts	0.114	4.841**	0.451	0.462	0.536	3.237**	0.087	0.086

Note: “*” and “**” represent significant and extremely significant difference from CK

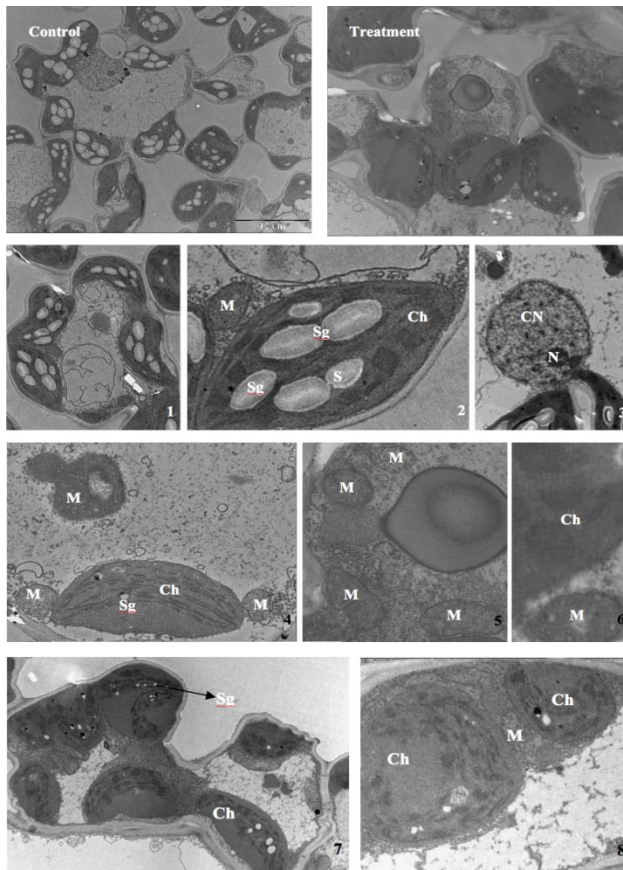


Fig. 3. Ultrastructure of Longjing 27 in the control group and Longjing 27 treated with 0.2% concentration alkali
 1. distribution of chloroplast, mitochondria and amyloplast in CK cells × 8000; 2. chloroplast, mitochondria and amyloplast in CK cells × 8000; 3. cell nucleus in CK × 10000; 4. chloroplast and mitochondria in treated one × 15000; 5. mitochondria in treated one × 20000; 6. chloroplastgrana amplifying; 7. distribution of organelles in treated cells × 10000; 8. chloroplast in treated one × 5000. (M. mitochondria, CN. cell nucleus, N. nucleolus, Ch. chloroplast, Sg. amyloplast, S. starch grain. The same as below).

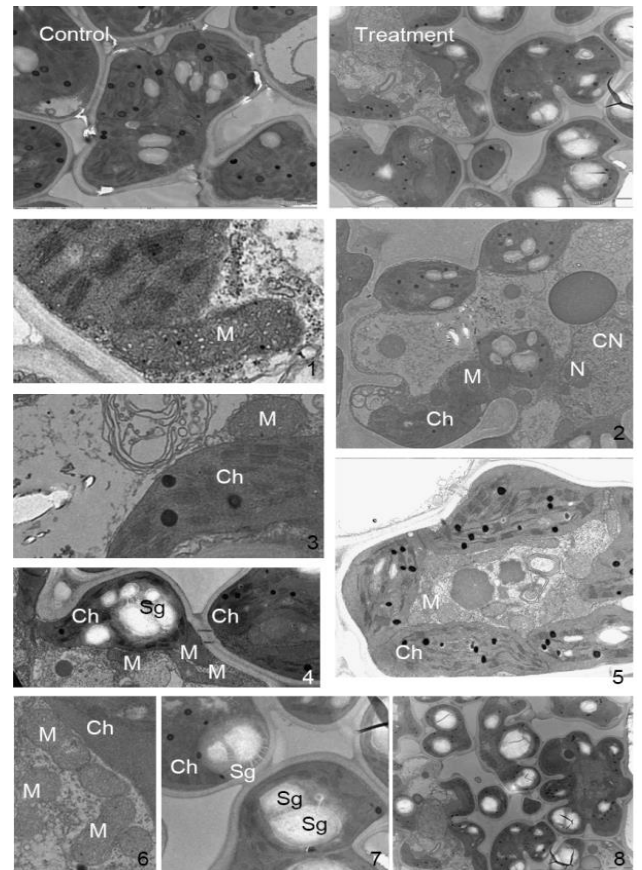


Fig. 4. Ultrastructure of CK Suijing 5 and one treated with 0.2% concentration alkali
 1. chloroplast and mitochondria in CK cells × 25000; 2. chloroplast, mitochondria and amyloplast in CK cells × 7000; 3. chloroplast, mitochondria and osmiophilic granule in CK cells × 20000; 4. distribution of chloroplast, mitochondria and proteosome in treated one × 12000; 5. distribution of cell organelle in treated one × 12000; 6. distribution of mitochondria nearby chloroplast × 8000; 7. big amyloplast in chloroplast in treated cells × 8000; 8. amyloplast in chloroplast × 5000.

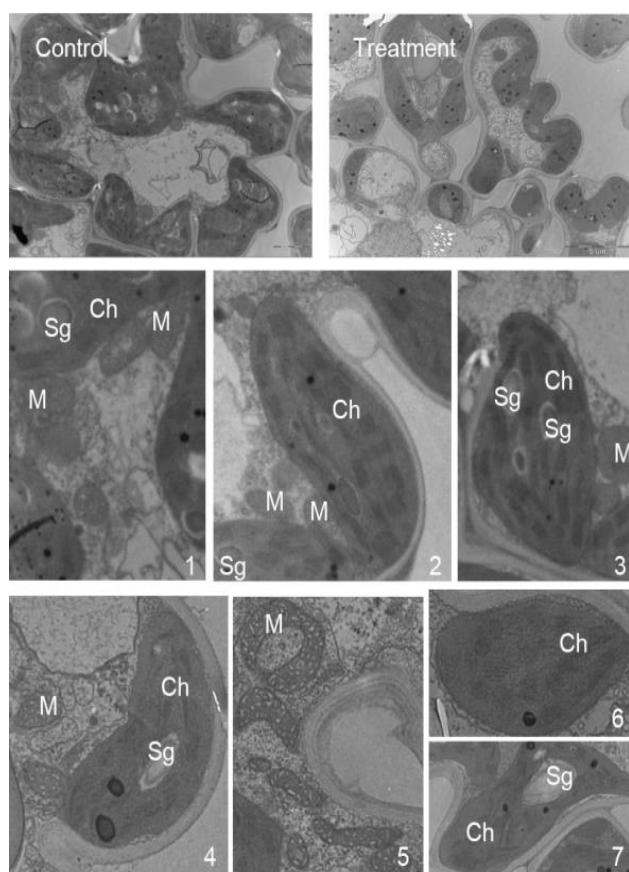


Fig. 5. Ultrastructure of CK Tengxi 138 and the one treated with 0.2% concentration alkali

1. chloroplast, mitochondria, amyloplast and osmiophilic granule in CK cells; 2. structure of chloroplast and mitochondria in CK cells; 3. chloroplast, mitochondria in CK cells; 4. chloroplast, mitochondria and proteosome in treated one; 5. mitochondria in treated one; 6. change of chloroplast grana in treated one; 7. big amyloplast in disordered chloroplast in treated one.

This analysis showed that the microscopic damage to the Lingjing 27 seedlings by alkali stress mainly occurred in the chloroplast and mitochondria. According to the internal structural changes of the chloroplast and the quantity and volume of the amyloplasts, the damage affected the formation of photosynthetic products. Although the number of mitochondria was increasing, due to the small number of inner ridges and swelling, the respiratory consumption was enhanced and its function was reduced. The photosynthetic products of chloroplast synthesis were fewer, the starch synthesis was reduced, and the transported sugar was also reduced such that the physiological functions of the plants were in a state of disorder and starvation. This will cause stunted growth, and eventually lead to the plants becoming withered.

Ultrastructure of leaf cells in Suijing 5: The structure of cell organs in Suijing 5 without alkali treatment was relatively clear (Fig. 4), and the mitochondria were linear, elliptical or circular, with a clearly visible ridge (Fig. 4-1). Its actual shape was rich, indicating a strong metabolism. Most of the chloroplasts were of fusiform or elliptical shape, had a relatively clear granule layer structure, and had a wide granule thickness. The number of stacked thylakoids

was high, and the spacing between two adjacent granules reasonable. The matrix layer between the connecting granules was relatively clear (Fig. 4-3) and the number of amyloplasts embedded in the chloroplast was high (Fig. 4-2). After treatment, the chloroplasts in the cells were rich in eosinophils (Fig. 4-5), the number of chloroplasts was large, the mitochondria differed in shape, and the inner ridge was abundant and distributed around the chloroplasts (Fig. 4-5, 6). The distance between the granules was not uniform, and the stroma-thylakoids between granules were relatively clear. Some chloroplast granules had a complex order, indicating that the damage was serious. Some chloroplasts contained large amounts of large starch granules (Fig. 4-8), which squeezed the chloroplast granules to the edges, but the granules were relatively clear. In some amyloplasts, there was a filamentous connection between the small starch granules and the large starch granules. The large starch granules were connected with the chloroplast membrane envelope and amyloplast membrane envelope. The proliferated small starch granules also continued to grow by the starch compound provided by the large starch granules. Our results showed that the synthesis and differentiation of starch in Suijing 5 was more intense after alkali treatment.

Compared with the control group, the amyloplast volume in the chloroplast of the treatment group was larger. This indicated that the alkali treatment had less effect on the activity of starch synthase in the chloroplast of Suijing 5, and the photosynthetic products were stored more than in the control group. The starch filamentous connection in Fig. 4-7 showed the functions of starch synthesis, storage, and differentiation were still strong. But the starch granules were too large, causing some chloroplasts to be seriously impaired indicating that the differentiation of amyloplasts was passive and the abnormal accumulation of photosynthetic products should be caused by the abnormal migration of sugar. The transportation of photosynthetic products in the chloroplast was inefficient, resulting in oversized amyloplasts. The mitochondrial ridge was relatively clear, indicating that the damage was lighter. This analysis showed that although the shape of chloroplasts in the leaves of Suijing 5 was significantly altered after alkali stress, it maintained a certain photosynthetic function. Altering the balance of photosynthetic product migration and synthesis was the main cause of physiological disorders. All of these results indicated that among the four tested rice varieties, Suijing 5 was least affected by alkali stress and is more resistant to both salt and alkali.

Ultrastructure of leaf cells in Fujisawa 138: A large number of mitochondria were distributed around chloroplast in the control group (Fig. 5-1), and its structure was normal with a relatively clear inner ridge. The shape of chloroplast in the control group was fusiform, and the granules were widely distributed with a normal structure. The granule thylakoids and stroma-thylakoids in the control group were clearly observable, and chloroplasts contained abundant amyloplasts (Fig. 5-2, 3). After treatment, a large number of mitochondria were distributed around chloroplast and its inner ridge was clear, indicating a strong metabolic function. However, the thickness of the granules in chloroplasts

was significantly thinner (Fig. 5-4) or swelling (Fig. 5-6). The granules were arranged in a disorderly fashion. The inlaid amyloplasts were reduced, the number of osmiophilic granules was increased, resulting the deepened osmium level.

After treatment with the alkali solution, the chloroplast granules in Fujisawa 138 were reduced, partial spheroidization occurred, disordered, and its number was decreased. The number of synthetic amyloplasts was decreased, but had little effect on mitochondrial structure and function, and migration of carbohydrates. In summary, the main effect of alkali stress on Fujisawa 138 led to the structural damage of chloroplasts thus reducing the photosynthetic function, with a subsequent reduction in synthetic products. This means there is an insufficient supply of raw materials for cell metabolism, the main cause of plant damage.

Ultrastructure of leaf cells in Koshihikari: In the control group, chloroplasts were inlaid with a large number of amyloplasts. Most of the chloroplasts were fusiform, and a large number of granules were orderly arranged. There were numerous mitochondria that varied in shape, and were distributed around the chloroplast (Fig. 6-2). Its inner ridge was relatively clear (Fig. 6-3), indicating that the metabolism was strong. The amount and volume of amyloplasts after alkali treatment was significantly greater than that of the control group. There were between one and three larger starch granules in each amyloplast, surrounded by a larger number of small amyloplasts (Fig. 6-4). After treatment, the volume of the amyloplasts was too large, causing the chloroplast to be nearly circular, the granules to be narrow and the thylakoids to be sparse (Fig. 6-5, 8). They were also widely distributed in the chloroplast. The number of mitochondria decreased significantly relative to the control group (Fig. 6-6, 9), and the number of inner ridges was decreased indicating that metabolic function was affected, and respiration was enhanced.

After alkali treatment, the thylakoids in chloroplasts changed greatly but retained their clear structure, so little effect on photosynthetic function was shown. According to the changes in the number and volume of amyloplasts, it was shown that the conversion of amyloplast to glucose was blocked. Mitochondria were seriously impaired by alkali stress. Therefore, the physiological characteristics of Koshihikari under alkali stress were as follows: the chloroplast structure was affected but photosynthesis was still ongoing, the transportation of sugar was blocked, and the structure and function of mitochondria were seriously impaired, resulting in photosynthetic synthesis. Finally, the photosynthetic product was accumulated in large quantities in the leaves, and the formation of the amyloplasts could not be transported resulting in hindered plant growth.

Discussion and conclusions

Effects of alkali stress on rice root cells: Under stress, the morphology of plant cells change accordingly and the meristems undergo abnormal differentiation (Wang *et al.*, 2017; Bonaventura *et al.*, 2005). It has been reported that under salt stress, the epidermis cells of *Arabidopsis thaliana*

become smaller (Skirycz *et al.*, 2011; Huang *et al.*, 1997). This study found that the length of parenchyma cells and stele cells significantly shortened, which was consistent with previous research. The suberization and lignification of the inner and outer cortical cells is considered to be an important feature of plant adaptation to stress. The roots of plants promote the transport of H₂O molecules through lignification and restrict the free migration of Na⁺, Ca²⁺ and Mg²⁺ ions to the outside to improve its resistance to stress (Yamauchi *et al.*, 1996). This study identified the lignification of cortical cells under alkali stress in different rice varieties, and found that rice changed its root structure to adapt to stress. The vascular bundle is the main water-transporting tissue. It has been found that some stress can promote the radial thickening of the vascular column, the deepening of lignification of the roots, and an increase in the number of vessels to improve the transport capacity of the roots (Bruns *et al.*, 1990). However, our study showed that under alkali stress, the number of large vascular bundles in rice roots was decreased, and the number of vascular bundles with incomplete development was increased. However, some images and data (Fig. 1-b-8) showed that the area proportion of vascular bundles to radiant roots was increased. It is thought that this is the result of an adaptive response of rice roots to the stress environment, which is consistent with some of the results reported in this study.

Effects of alkali stress on rice leaf subcellular structure:

When it comes to microstructural changes of plant cells under stress, researchers agree that stress can degrade or destroy the ultrastructures of chloroplasts, mitochondria, and other organelles (Li *et al.*, 2015; Ashraf & Harris, 2004). The results of this study indicate that alkali stress can cause the deformation of chloroplast and the swelling of granules or reduction of the number of lamellae. However, these effects vary depending on the variety of rice, which is consistent with previously reported research (Yang *et al.*, 2016). The change of ultrastructure is a complicated and dynamic process. Maintaining the balance of physiological functions under stress should be the fundamental cause of microstructural changes. Therefore, it cannot be characterized by a single change in the number, shape or position of an organelle. In this study, we observed and analyzed the rice varieties from China and Japan, and found that the changes in microstructure had comprehensive or integral characteristics. There are similarities and differences in these characteristics across different rice varieties.

Chloroplasts are the organelles most sensitive to stress. The main changes of chloroplasts identified by this study were that the lamellar structure of the granule thylakoids was reduced and some of the thylakoids connected by stroma were arranged in a disorderly fashion (Liu *et al.*, 2007). There were also differences in the structural changes of chloroplasts between varieties, and the vesicles of some were biased toward the side of the chloroplast. The thylakoids of some varieties were biased to the side of the chloroplast (Longjing 27), while changes in the position of the thylakoids of other rice varieties (Suijing 5, Fujisawa 138, Koshihikari) were not obvious. The number and structure of mitochondria also varied between rice varieties. The number of

mitochondria in Longjing 27 was increased, the swelling was obvious, and the inner ridge was less dominant indicating that respiration was enhanced and the consumption increased accordingly. The tricarboxylic acid cycle and oxidative phosphorylation pathway could not function properly. Meanwhile, the volume of synthetic starch granules was greatly decreased, indicating that the chloroplast function was seriously impaired. The photosynthetic product 3-phosphoglycerate decreased, the physiological functional efficiency of the organism seriously degraded, and the overall function of the plant was weak. Under stress, a large number of large-sized starch granules were stored in the chloroplasts of Suijing 5. Similar phenomena has been observed in some other studies (Suriyan *et al.*, 2009), providing a basis for judging the stress resistance of plants.

After treatment, there were some filaments between some starch granules and the cell membrane. Under stress, the structure of chloroplasts was destroyed, and the photosynthetic function suffered, leading us to suspect that the saccharides derived from photosynthesis could not normally be exported for rapid conversion of synthetic starch and result in a large accumulation of starch in the chloroplast to form starch granules. Large starch granules transported the transformed sugar to the cell membrane via filaments. After the alkali treatment, the membrane system was seriously impaired (Su *et al.*, 2011). Most of the sugar was transported to form starch granules or differentiated small starch granules, but the process causing the large accumulation of starch granules was passive. Stress treatment resulted in a large number of mitochondria, which were slightly swollen. This indicated that the respiration process was strengthening. The change in osmiophilic granules may be caused by the large accumulation of lipid substance in the chloroplast or vacuole due to degradation of the chloroplast thylakoid membrane.

The mitochondria in Fujisawa 138 maintained its normal structure after stress treatment, but the structure of the chloroplast was seriously impaired. The granule thylakoids disintegrated (Fig. 4-5-6), indicating that photosynthesis was not occurring properly and other starch granules were stored. In the chloroplast, the thylakoid structure was severely distorted and the larger amyloid was located in the center of the chloroplast, and might have lost its transformation function. Despite the normal morphology and function of mitochondria, there is a lack of energy supply. After stress treatment, both the number and volume of amylose in the chlorophyll green color was significantly larger than that of the control. This resulted in the shape of the chloroplast being nearly round, while the thylakoid layer was reduced and twisted. This was similar to Suijing 5, however, there is no filament phenomenon in Koshihikari. Therefore, the conversion process between amylose and sugar was weaker than that of Suijing 5, and the surface area of ray and granules increased significantly after treatment. However, the structure was ambiguous, indicating that respiratory consumption increased and functional damage was heavier. Despite the fact that chloroplast photosynthesis was still ongoing, carbohydrate conversion and photosynthetic product transport were blocked.

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References

- Ashraf, M and P.J.C. Harris. 2004. Potential biochemical indicators of salinity tolerance in plants. *Plant Sci.*, 166(1): 3-16.
- Barkla, B.J., R. Vera and O. Pantoja. 2013. Progress and challenges for abiotic stress proteomics of crop plants. *Proteomics*, 13(13): 1801-1815.
- Bonaventura, R., V. Poma, C. Costa and V. Matranga. 2005. UV-B radiation prevents skeleton growth and stimulates the expression of stress markers in sea urchin embryos. *Bioch. & Biophys. Res. Comm.*, 328 (1): 150-157.
- Bruns, S and C. Hecht-Buchholz. 1990. Light and electron microscope studies on the leaves of several potato cultivars after application of salt at various development stages. *Potato Res.*, 33(1): 33-41.
- Chinnusamy, V., A. Jagendorf and J.K. Zhu. 2005. Understanding and improving salt tolerance in plants. *Crop Sci.*, 45(2): 437-448.
- Flowers, T.J and A.R. Yeo. 1995. Breeding for salinity resistance in crop plants: where next? *Fun. Plant Biol.*, 22: 875-884.
- Geissler, N., S. Hussin and H.W. Koyro. 2009. Elevated atmospheric CO₂ concentration ameliorates effects of NaCl salinity on photosynthesis and leaf structure of *Aster tripolium* L. *J. Exp. Bot.*, 60(1): 137-151.
- Huang, Z.Y., H. Wu and Z.H. Hu. 1997. The structures of 30 species of psammophytes and their adaptation to the sandy desert environment in xinjiang. *Acta. Phytocol. Sinica*, 21(6): 521-530.
- Jiang, Y. and B. Huang. 2001. Drought and heat stress injury to two cool-season turf grasses in relation to antioxidant metabolism and lipid peroxidation. *Crop Sci.*, 41(2): 436-442.
- Kosová, K., P. Vítámvás and I.T. Prášil. 2011. Plant proteome changes under abiotic stress contribution of proteomics studies to understanding plant stress response. *J. Prot.*, 74(8): 1301-1322.
- Lei, S., S. Zhao and G. Xia. 2006. Research progress on the identification of salt-tolerance related genes and molecular mechanism on salt tolerance in higher plants. *Mol. Plant Breed.*, 4(1): 15-22.
- Li, H., Q. Sun, S.J. Zhao and W.H. Zhang. 2000. *Principles and techniques of plant physiological biochemical experiment*. Higher Education, Beijing, 195-197.
- Li, X.F., Z.M. Ni, Y.Y. Wang, M.Q. Li, R. Liu and H.Y. Rao. 2015. Effects of salt stress on photosynthetic characteristics and leaf cell structure of Yinhong grape seedlings. *Acta. Ecol. Sin.*, 35(13): 4436-4444.
- Liu, Y.J., X.Y. Tian and M.J. Cao. 2007. The effects of low potassium stress on root growth and potassium absorption of maize seedling. *J. Maize Sci.*, 15(2): 107-110.
- Miyake, H., S. Mistsuya and M.S. Rahman. 2006. Ultrastructural effects of salinity stress in higher plants. *Abiotic Stress Toler. Plants*, 113: 215-226.
- Mukherjee, A., S.P. Acharya and M.S. Janaki. 2021. Dynamical study of nonlinear ion acoustic waves in presence of charged space debris at Low Earth Orbital (LEO) plasma region. *Astroph. & Space Sci.*, 366(1): 11-14.

- Negrão, S., A.M.C. Almadanim, I.S. Pires, I.A. Abreu, J. Maroco, B. Courtois and O.M. Margarida. 2013. New allelic variants found in key rice salt tolerance genes: an association study. *Plant Biotechnol. J.*, 11(1): 87-100.
- Petrov, V., J. Hille, B. Mueller-Roeber and T.S. Gechev. 2015. ROS-mediated abiotic stress-induced programmed cell death in plants. *Front. Plant Sci.*, 6: 69-85.
- Qing, K.Y. and P.T. Guo. 1999. Effects of salt stress on the ultrastructure of chloroplast and the activities of some protective enzymes in leaves of sweet potato. *Acta Phytology. Sinica*, 25(3): 229-233.
- Skiryecz, A., H. Claeys, S.D. Bodt, A. Oikawa, S. Shinoda, M. Andriankaja and K. Saito. 2011. Pause-and-stop: the effects of osmotic stress on cell proliferation during early leaf development in Arabidopsis and a role for ethylene signaling in cell cycle arrest. *The Plant Cell*, 23: 1876-1888.
- Su, F.L., H.F. Li, X. Chen, J.Q. Chen and T.L. Wang. 2011. Effect of salt stress on the ultrastructure of reed cell. *Acta Bot. Boreali-Occidentalia Sinica*, 31(11): 2216-2221.
- Suriyan, C., S.W. Kanyaratt and K. Chalermopol. 2009. Comparative effects of salt stress and extreme pH stress combined on glycinebetaine accumulation, photosynthetic abilities and growth characters of two rice genotypes. *Rice Sci.*, 16(4): 274-282.
- Wang, Z., J.B. Liu, M.L. Chen, G.X. Su, B.C. Xu and J. Huang. 2017. Diurnal and seasonal variations in photosynthesis, leaf water potential and soil water content of six alfalfa cultivars on the semiarid Loess Plateau. *Pak. J. Bot.*, 49(5): 1717-1724.
- Xiong, L. and J.K. Zhu. 2002. Molecular and genetic aspects of plant responses to osmotic stress. *Plant. Cell & Environ.*, 25(2): 131-139.
- Yamane, K., M. Kawasaki, M. Taniguchi and H. Miyake. 2008. Correlation between chloroplast ultrastructure and chlorophyll fluorescence characteristics in the leaves of rice (*Oryza sativa* L.) grown under salinity. *Plant Prod. Sci.*, 11(1): 139-145.
- Yamauchi, A., J.R. Pardales and Y. Kono. 1996. Root system structure and its relation to stress tolerance. *Roots and Nitrogen Crop. Syst. Semi-Arid Trop.*, 16: 211-233.
- Yang P., F. Zhou, B.J. Zhang, L.K. Wang, B.L. Qian and Q.Z. Chen. 2016. Phylogenetic analysis and detection of selective pressure on plant salt overly sensitive genes. *Mol. Plant Breed.*, 8: 2098-2105.
- Zhao, G.M., Q.M. Shi, Y. Han, S.H. Li and C.H. Wang. 2014. The physiological and biochemical responses of a medicinal plant (*Salvia miltiorrhiza* L.) to stress caused by various concentrations of NaCl. *Plos One*, 9(2): 896-901.
- Zhu, Y.J., Y. Zhang, Z.Z. Hu and S.G. Yan. 2000. Studies on microscopic structure of *Puccinellia tenuiflora* stem under salinity stress. *Grassland of China*, 5: 6-9.

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