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RESEARCH ARTICLE

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Cytochemical localization of H^+-ATPase and sub-cellular variation in mesophyll cells of salt-treated *Cyclocarya paliurus* seedlings

Abstract *Cyclocarya paliurus* seedlings of three provenances were treated with three levels of salt (0, 51.0 and 85.0 mmol/L NaCl) in a hydroponic system of a phytotron. The ultrastructural distribution of H^+-ATPase activity and ultrastructural variation were investigated in mesophyll cells of *C. paliurus* seedlings after 12 or 20 days of salt treatment. The results showed that under normal growing conditions, H^+-ATPase activity was low and localized mainly in the nucleus. After 12 days of salt treatment and an increase in salinity, we found that the greater the H^+-ATPase activity, the lighter the damage suffered by mesophyll subcells after 20 days of salt treatment and the stronger the salt-adaptation ability of seedlings. The location of H^+-ATPase, largely in the nucleus, indicated that salt-damage suffered by the seedlings was light, whereas its presence, mainly in the vacuoles, showed that salt-damage was more serious. Our preliminary conclusion is that the salt-tolerance level of *C. paliurus* seedlings for the three kinds of provenances was in the following order, from high to low: Huangshan seedlings from Anhui Province > Kunming seedlings from Yunnan Province > Jiujiang seedlings from Jiangxi Province.

Keywords H^+-ATPase, ultrastructure, NaCl, mesophyll cell, *Cyclocarya paliurus*, cytochemistry

1 Introduction

The enzyme ATPase occurs extensively in organisms and has a wide range of important functions, including catalyzing the hydrolyzation of ATP, participating in the transport of material, ions and cellular signal transduction and many kinds of metabolizations (Joshi et al., 1988; Hasegawa et al., 2000; Chen et al., 2006; Christian et al., 2006). At present, three kinds of ATPase are often seen in plant cells: H^+, Ca^{2+} and Mg^{2+}-ATPase. H^+-ATPase plays a central role in maintaining the pH of cytoplasm, the Ca^{2+} concentration, water potential and the stabilization of intracellular metabolization (Martinez-Ballesta et al., 2003; Salem et al., 2005). Cytochemical methods are powerful tools for the precise mapping of the distribution of ATPase and are able to localize its activity at the ultrastructural level *in situ* (Wang and Sze, 1985; Morsomme and Boutry, 2000; Wang et al., 2001; Shi et al., 2003; Qiu et al., 2004; Yang et al., 2007). Still, at present, very little is known about the distribution and the activity localization of this enzyme in woody plants under adverse stress conditions (Yang et al., 2007).

Cyclocarya paliurus (Batal.) *iljinskaja* (family Juglandaceae) is native to China and the sole species in its genus and mainly grows from 420–2500 m in elevation in mountainous regions. In China, the bark and leaves of *C. paliurus* are widely used for making medicinal tea (Xie and Li, 2001). A new crystalline compound, called cyclocaric acid A (3, 23-*β*-epoxy-olean-12-en-28-oicacid), was isolated from the leaves of *C. paliurus* (Fang and Fu, 2007) and a rich amount of polysaccharides from the leaves of *C. paliurus* was confirmed, which is effective in reducing blood glucose and improves the capacity of glucose tolerance in diabetic mice (Xie and Li, 2001; Li et al., 2002). An *in vitro* study showed that *C. paliurus* inhibits alpha-glucosidase, a disaccharide-degrading enzyme in the small intestinal mucosa, leading to a decrease in the absorption of 4-glucose into the blood and a subsequent lowering of the blood glucose level (Kurihara et al., 2003). Xie and Li (2001) reported that the amounts of flavonoid, vitamin E (VE) and vitamin C (VC) in the leaves of *C. paliurus* were also greater than in other plants. An enormous production of tender leaves is required if it is

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to be used as raw material from *C. paliurus* for tea and medical use and this large demand for leaf production will require the establishment of new plantations of *C. paliurus*.

On a worldwide scale, there is an area of around 380 million hm^2 that is potentially usable for agriculture, but where production is severely restricted by salinity (Lambers, 2003). In China, there are about 27 million hm^2 of saline soils, of which coastal lands account for 8% (Yao and Fang, 2007). Therefore these land resources would present a great potential for forest development. To our knowledge, no information is available on the salt tolerance of *C. paliurus* at different stages of its life cycle. Since the mechanism by which plants tolerate salt is complex and differs from species to species (Greenway and Munns, 1980; Ashraf and Mcneilly, 1987), the objectives of this study are: 1) to investigate the H^+-ATPase distribution and the localization of this enzyme, its activity and ultrastructural variation; and 2) to evaluate the salt-tolerance of *C. paliurus* seedlings. The results from this study will contribute to an enhanced understanding of the relation between the H^+-ATPase and salt-tolerance of *C. paliurus* and provide a theoretical and fundamental basis for site selection and plantation silviculture.

2 Materials and methods

2.1 Plant material and growth conditions

Seeds of *C. paliurus* were collected in October 2005 from Huangshan in Anhui Province (A), Jiujiang in Jiangxi Province (J) and Kunming in Yunnan Province (Y), China. Seed trees were about 15 m tall and 28 cm in diameter at breast height. After drying in open sunlight and removal of unwanted material, the seeds were treated with condensed sulphuric acid and rinsed with water. The seeds were stratified in pails according to a volume ratio of 1 (seed) to 3 (sand, mixed with gibberellins) under natural conditions in order to break seed dormancy. The stratification was started in January 2006 and ended in March 2006. Seeds were sown in containers with a mixed medium (perlite: vermiculite:peat soil = 1:2:2) after they were stratified. When the seedlings were about 7 cm tall, they were transplanted to black plastic boxes with a half-strength Hoagland nutrient solution. The dimensions of the plastic boxes were 56 cm (length)×36 cm (width)×26 cm (height). The plastic was 8 mm thick. After 10 days of cultivation, uniform seedlings were selected and placed in plastic boxes with a Hoagland nutrient solution containing different concentrations of NaCl.

NaCl treatments were started in July 2006 with three levels of NaCl: 0.0 (CK, i.e. control), 51.0 (S1) and 85.0 (S2) mmol/L of NaCl, respectively. In total, the following nine treatments were designed: ACK, AS1, AS2, JCK, JS1, JS2, YCK, YS1 and YS2. Each treatment consisted of 45 seedlings. The solution was aerated throughout the experiment, the volume was maintained by adding water to compensate for water loss from evaporation and transpiration and the nutrient solution was renewed every seven days since the seedlings were transplanted to the boxes. The seedlings were grown in a phytotron at a photon flux density of 350–400 μmol/($m^2 \cdot s$) for 12 hours each day, at temperatures of 26±0.5/20±0.5°C and relative humidity of 70%/80% in day/night cycles.

2.2 Cytochemical localization of H^+-ATPase

The ultrastructural enzyme cytochemical technique involved the use of a heavy metal simultaneous capture reaction (Joshi et al., 1988) and was a modification to that reported by Chen et al. (2006). Briefly, after 12 days of salt treatment, leaves at the same position were cut into fractions about 0.3 mm^2, and rapidly placed in 2.5% glutaric dialdehyde and 4% paraformaldehyde, fixed for 2 h at 4°C, washed four times (half an hour each) with a 50 mmol/L cacodylate buffer and the remaining glutaric dialdehyde was washed with a 50 mmol/L tris-maleate buffer, pH 7.2, then incubated for 2 h at 37°C. The incubation medium was made up of a 50 mmol/L tris-maleate buffer (pH 7.2), 2 mmol/L ATP (Na^+ salt), 3 mmol/L $Pb(NO_3)_2$, 5 mmol/L $MgSO_4 \cdot 7H_2O$ and 1% sucrose. For the control plants, the incubation medium was without the addition of ATP (Na^+ salt). After a 2-hour incubation, the samples were washed twice at 4°C with a 50 mmol/L tris-maleate buffer (pH 7.2), rinsed briefly with a 50 mmol/L cacodylate buffer, pH 7.2 and fixed with 1% osmium tetroxide confected with a 0.1 mol/L cacody-late buffer (pH 7.2) for 2 hours at 4°C, then washed four times with a 50 mmol/L cacodylate buffer. Finally, samples were dehydrated in an alcohol gradient, passed through propylene oxide, embedded in Epon812 and then sliced using a LKB-2088 type ultramicrotome. Sections were investigated without a uranium and lead citric acid acetate stain and photographed with a transmission electron microscope of the HITACHI H-600 type.

2.3 Investigation of ultrastructural variation

The investigation of ultrastructural variation was conducted as described by Peng et al. (2004) with a few modifications. After 20 days of salt treatment, fractions about 1 mm^2 of leaves collected from the same position were quickly put into 3% glutaric dialdehyde with a 0.1 mol/L phosphoric acid buffer, pH 7.0, fixed for 3 h at 4°C, washed three times (half an hour each) with the same buffer, then fixed in 1% osinic acid confected with a 0.2 mol/L phosphoric acid buffer (pH 7.0) for 24 h at 4°C, then washed three times with the same buffer solution. After a wash in this buffer solution, samples were dehydrated in an alcohol gradient, passed through propylene oxide, embedded in Epon812, then sliced using a LKB-2088 type ultramicrotome. Sections were

stained with uranium acetate and lead citric acid, observed and photographed with the HITACHI H-600 type transmission electron microscope.

3 Results

3.1 Cytochemical localization of H^+-ATPase in mesophyll cell under salt treatment

In order to investigate clearly the distribution of H^+-ATPase and not to confuse it with electric staining or artificial feint, H^+-ATPase activity could be verified only by comparison with the control. The results by comparison with the controls with or without the addition of ATP (Na^+ salt) showed that no lead phosphate deposits were found in the control without the addition of ATP (Na^+ salt) (Figs. 1a,e,i), while lead phosphate deposits were found without diffusing phenomena in the control plants with the addition of ATP (Na^+ salt) (Figs. 1b,f,j). This indicated that the results, shown in the figures, were credible.

After 12 days of salt treatment, cytochemical localization of H^+-ATPase in *C. paliurus* from the different provenances showed that, in *C. paliurus* from the Hunshan provenance, H^+-ATPase activity was mainly found on the karyon, endoplasmic reticulum and golgi apparatus and that, with the increase of salinity, H^+-ATPase activity increased (Figs. 1b,c,d). In the Kunming provenance, H^+-ATPase activity on the vacuole and plasmalemma was greater; with an increase in salinity, H^+-ATPase activity on the vacuole and osmiophilic globule on the thylakoids became significantly greater (Figs. 1j,k,l). H^+-ATPase activity in *C. paliurus* from Jiujiang was mostly found on the vacuole; the higher the salinity, the greater the H^+-ATPase activity. However, the H^+-ATPase activity of the Jiujiang provenance was generally less than that of the Huangshan and Kunming provenances (Figs. 1f,g,h). These results indicated that the H^+-ATPase activity in mesophyll cells of *C. paliurus* seedlings is related to provenance and salinity.

3.2 Ultrastructural variation of leaves under salt treatment

From Fig. 2, we see that, after 20 days of salt treatment, with a gradual increase in salinity, damage suffered by seedlings of the three provenances increased. Generally, variation in the ultrastructure of leaves was involved in the disappearance of chloroplast membranes, the swelling of thylakoids and even in the degradation of chloroplast. In contrast with the control plants, the starch particles on the chloroplast clearly decreased, while the number of osmiophilic globules increased. At the same time, the karyotheca vanished and the karyotin appeared to condense, simultaneously, accompanied by the occurrence of plasmolysis. In this study, given the same level of salt stress, damage of leaf subcells of the Huangshan provenance was the lowest, while that of the Jiujiang provenance was the most serious.

4 Discussion

The H^+-ATPase is a proton pump with a major role in the coupling of ATP hydrolysis in the transport of protons (Porillo, 2000; Kuhlbrandt, 2004). Regulation of the H^+-ATPase activity could represent an important cellular mechanism for salinity tolerance (Kalampanayil and Wimmers, 2001; Kerkeb et al., 2001; Zhao et al., 2004). An enhancement of the H^+-ATPase activity was observed in *C. paliurus* mesophyll cells growing on a medium containing NaCl. Reinhold et al. (1984) suggested that the H^+-ATPase may act as both a detector and an effective agent in response to salt stress. The H^+-ATPase is encoded by a multigene family and at least ten isoforms can be found in plants (Baxter et al., 2003). Krysan et al. (1996) analyzed T-DNA knockout *Arabidopsis* mutants of H^+-ATPase isoforms and demonstrated that at least one H^+-ATPase isoform is involved in NaCl tolerance. Modulation of this enzyme might not only occur at the enzyme level, but transcriptional and translational regulations may also be involved (Morsomme and Boutry, 2000). Salinity stress can induce mRNA accumulation and/or increase the amount of H^+-ATPase in some plants (Kalampanayil and Wimmers, 2001; Sibole et al., 2005). The H^+-ATPase was used in this study to determine the changes of this enzyme activity in *C. paliurus* of the three provenances exposed to salinity stress. Based on our results, the increase in H^+-ATPase activity was induced in NaCl-treated *C. paliurus* of the three provenances, which showed that the increase in H^+-ATPase activity in the Huangshan provenance was the greatest, while that of the Jiujiang provenance was the smallest under NaCl stress, i.e., the capacity of the Huangshan provenance to regulate the H^+-ATPase activity in response to NaCl stress was stronger than in the other two provenances of *C. paliurus*.

The response of H^+-ATPase to NaCl stress was involved in the change of the H^+-ATPase activity as well as the variation of the H^+-ATPase localization (Mi et al., 2006; Fang and Fu, 2007). In our study, we concluded that the H^+-ATPase was mainly found on the nucleus under natural growing conditions. However, with an increase in salinity stress, the location of H^+-ATPase gradually transferred to the vacuole.

From our results with the 51.0 and 85.0 mmol/L NaCl treatment, we conclude that the H^+-ATPase of the Huangshan provenance was mostly found on the nucleus and the damage suffered by the ultrastructure of leaves was slight, while the damage to the Jiujiang and Kunming provenances was mainly found on the vacuoles. The level of damage to the leaf ultrastructure was becoming more serious, suggesting that the damage, suffered by seedlings, was smaller when the H^+-ATPase was located in the

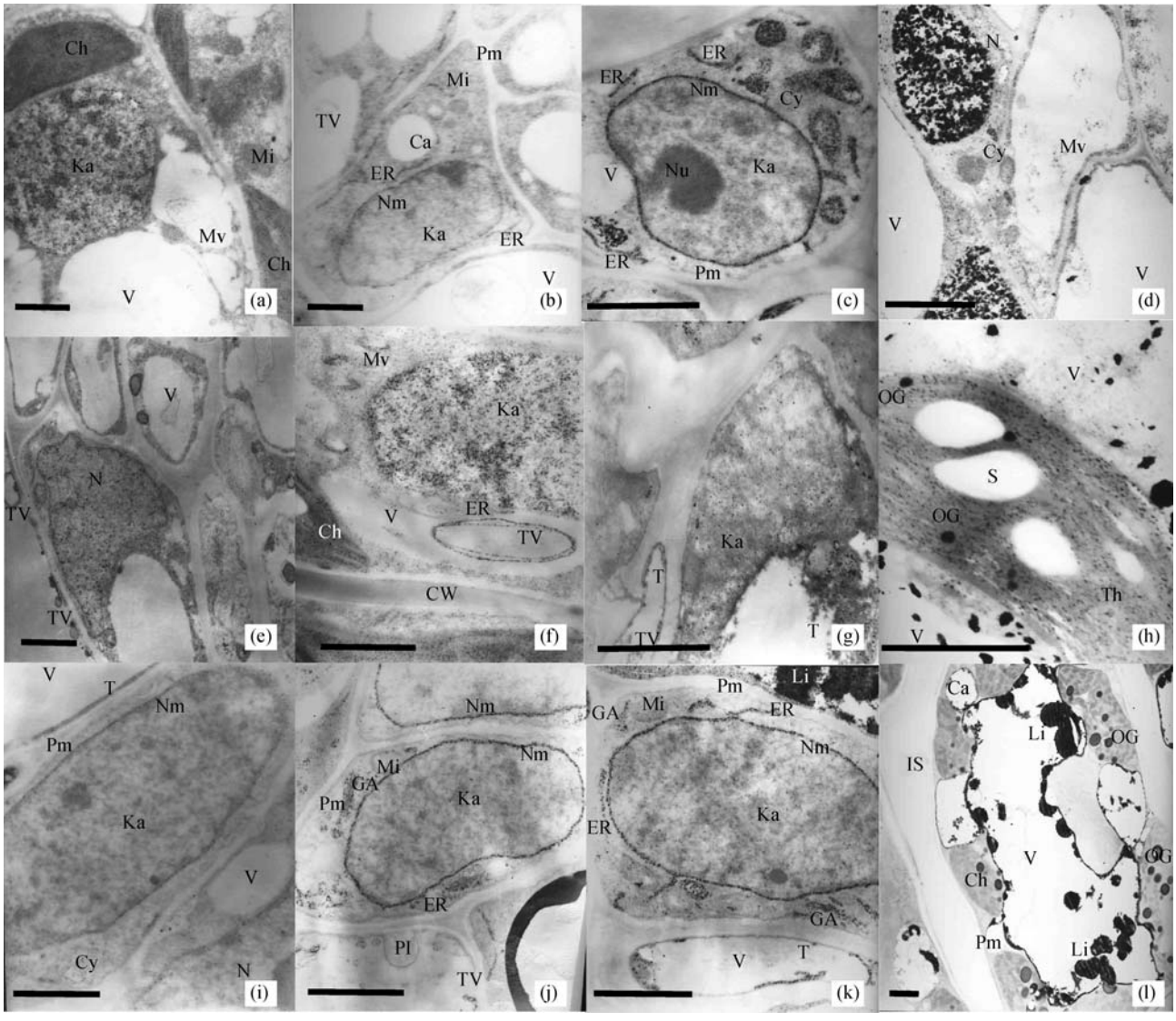

Fig. 1 Localization of H^+-ATPase activity in mesophyll cells of *C. paliurus* seedlings after 12 days of salt stress. (a) No H^+-ATPase activity reaction in mesophyll cells for the ACK treatment without substrate addition. (b) H^+-ATPase activity reaction on nuclear membrane and endoplasmic reticulum of mesophyll cells for ACK treatment. (c) A significant H^+-ATPase activity reaction on nuclear membrane and endoplasmic reticulum of mesophyll cells for the AS1 treatment. (d) Considerable reaction by H^+-ATPase activity on the nucleus of mesophyll cells for the AS2 treatment. (e) No H^+-ATPase activity reaction in mesophyll cells for the JCK treatment without substrate addition. (f) H^+-ATPase activity reaction on the endoplasmic reticulum, transfer vesicle, multivesicle bodies and nucleus of mesophyll cells, also for the JCK treatment. (g) A small amount of H^+-ATPase activity reaction on the transfer vesicle and tonoplast, while the H^+-ATPase activity was rarely found on the nucleus of mesophyll cells for the JS1 treatment. (h) Obvious grains of H^+-ATPase activity reaction on the vacuole of mesophyll cells for the JS2 treatment. (i) No H^+-ATPase activity reaction in mesophyll cells for the YCK treatment without substrate addition. (j) H^+-ATPase activity reaction on the endoplasmic reticulum, golgi apparatus, nuclear membrane of mesophyll cells for the YCK treatment and appearance of a phenomenon of plasmalemma invagination. (k) H^+-ATPase activity reaction on the endoplasmic reticulum, golgi apparatus, nuclear membrane and tonoplast of mesophyll cells for the YS1 treatment. (l) H^+-ATPase activity reaction on the tonoplast, high-density electronic substance in the cytochylema and more and larger osmiophilic globules on the thylakoids of mesophyll cells for the YS2 treatment. Explanation of abbreviations used: H^+-ATPase: H^+-Adenosine triphosphatase; AL: annulate lamella; Ca: cavum; Ch: chloroplast; CW: cell wall; Cy: cytoplasm; ER: endoplasmic reticulum; GA: golgi apparatus; Is: intercellular space; Ka: karyotin; Li: lipid; Mi: mitochondria; Mv: multivesicle bodies; N: nucleus; Nm: nuclear membrane; Nu: nucleolus; OG: osmiophilic globule; Pd: plasmodesmata; Pi: pit; PI: plasmalemma invagination; Pm: plasmalemma; S: starch grains; SW: secondary wall; T: tonoplast; TV: transfer vesicle; Th: thylakoids; V: vacuole. A, J and Y show Huangshan provenance from Anhui, Jiujiang provenance from Jiangxi and Kunming provenance from Yunnan, while CK, S1and S2 respectively show control, 51.0 and 85.02 mmol/L NaCl treatment. Bar = 1 μm (the same explanations apply to Fig. 2).

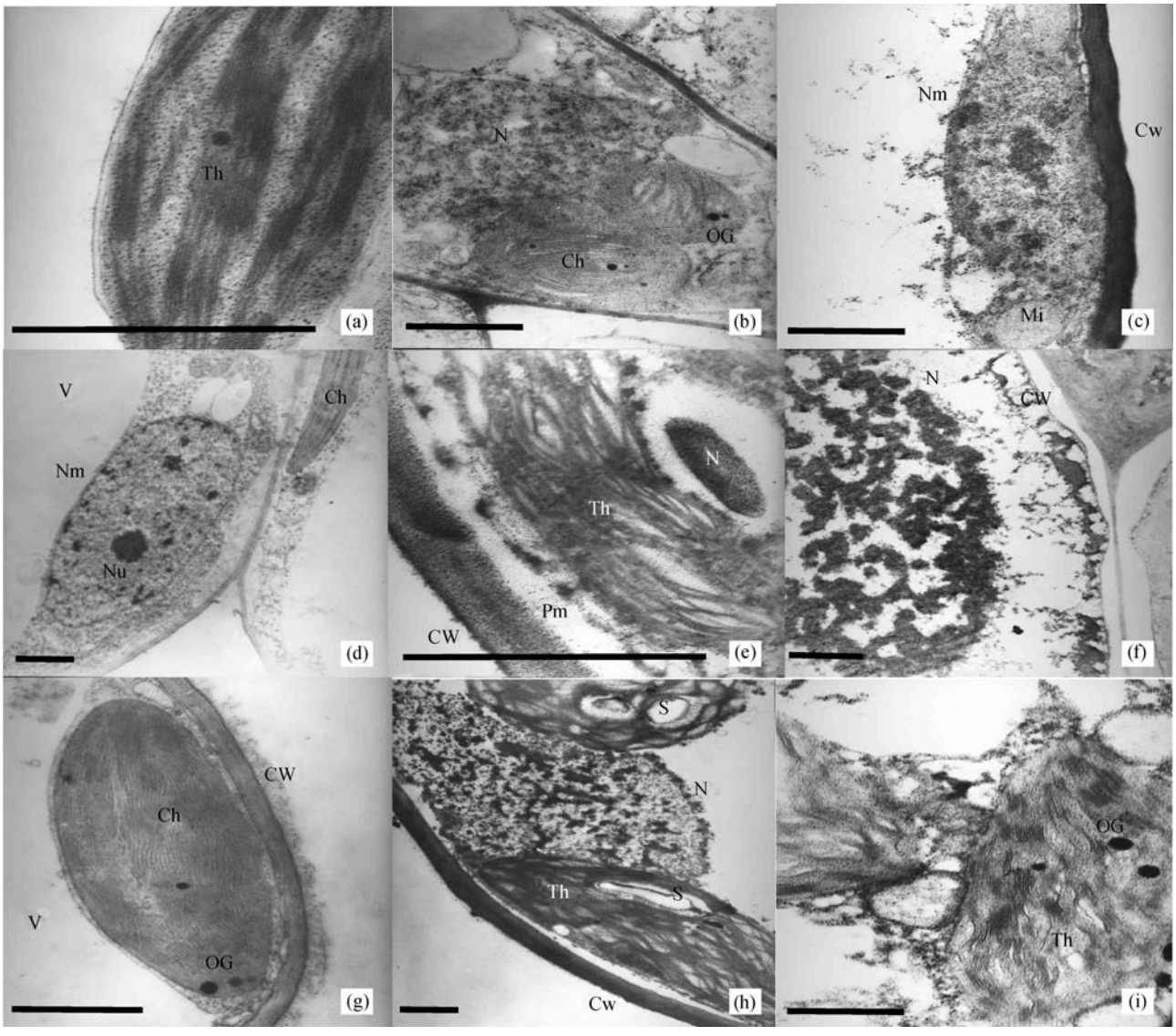

Fig. 2 Variation of leaf ultrastructure of *C. paliurus* seedlings after 20 days of salt stress. (a) the thylakoids of mesophyll cells for the ACK treatment. (b) the nucleus and chloroplast of mesophyll cells of the AS1 treatment. (c) the nuclear membrane, cell walls, multivesicle bodies and the plasmolysis phenomenon of mesophyll cells for the AS2 treatment. (d) the nucleolus, the nuclear membrane, chloroplast and vacuole of mesophyll cells for the JCK treatment. (e) thylakoids, the nuclear, cell walls, plasmalemma and plasmolysis phenomenon of mesophyll cells for the JS1 treatment. (f) the nucleus and cell walls of mesophyll cells for the JS2 treatment. (g) chloroplast, vacuole and cell walls of mesophyll cells for the YCK treatment. (h) thylakoids, nucleus and cell walls of mesophyll cells for the YS1 treatment. (i) thylakoids and osmiophilic globules of mesophyll cells for the YS2 treatment.

nucleus, while the damage was greater when the H^+-ATPase was located on the vacuole. Perhaps this is important for an early distinction of salt-tolerant species to provide a certain theoretical base.

Osmiophilic globules are considered an index of the function of some lipids, which often causes loss of plasmalemma functions (Zhang, 2006). An earlier report indicated that changed osmiophilic globules were connected to the level of damage of plants suffering under conditions of adverse stress (Sam et al., 2003). In our study, we found that the H^+-ATPase of the Kunming provenance was rarely found on the nucleus, while the number of its osmiophilic globules was considerably greater than the that of the other two provenances under the same level of NaCl stress, suggesting that its lipids were increasingly more efficient in protecting the integrity of plant plasmalemma, concluding that the capacity for salt-tolerance of the Kunming provenance was only inferior to the Huangshan provenance. In addition, given the 85.0 mmol/L NaCl treatment, the ultrastructure of leaves of *C. paliurus* of the three provenances was seriously damaged, the osmiophilic globules became considerably larger and their number increased compared with the control plants. These results implied that osmiophilic

globules could act as a double identifying index for judging the plant's capacity for salt-tolerance as well as the level of damage to plants suffering under NaCl stress.

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