BIOLOGIA PLANTARUM 50 (3): 469-472, 2006

BRIEF COMMUNICATION

Deposition pattern of hydrogen peroxide in the leaf sheaths of rice under salt stress

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Abstract

Deposition pattern of hydrogen peroxide (H_2O_2) under salt stress (100 mM NaCl) was examined cytochemically in rice (*Oryza sativa* L. cv. Pokkali) through the reaction of H_2O_2 with cerium chloride ($CeCl_3$) to produce electron dense precipitates of cerium perhydroxide. The distribution pattern of cerium perhydroxide precipitates in leaf sheath was considerably different from other parts of rice under salinity stress. Cerium perhydroxide precipitates were mainly accumulated on the tonoplast of leaf sheath under salinity, although they were localized on the cell wall and plasma membrane in all other tissues such as leaf blade and root.

Additional key words: cerium chloride, cytochemistry, energy dispersive X-ray analysis, *Oryza sativa*, transmission electron microscopy.

Salinity affects plant growth and crop productivity (Lin and Kao 1995, Agarwal and Pandey 2004). Although considerable effort has been done in the selection of salt tolerant (Pokkali) or susceptible (IR-29) rice cultivars (Lee *et al*. 2003), progress seems slow due to inadequate understanding of the mechanism of salt stress. Salinity stress in plants involves in the production of the reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) (Dionisio-Sese and Tobita 1998, Mittler 2002, Vaidyanathan *et al.* 2003). These ROS can seriously disrupt metabolism through oxidative damage to membranes of lipids, proteins, and nucleic acids (Dionisio-Sese and Tobita 1998).

To understand better these mechanisms, it is necessary to study the spatial distribution of ROS at cellular level under salt stress. Although a number of biochemical works have been done on ROS (Hilal *et al*. 1998, Vaidyanathan *et al.* 2003, Sairam and Tyagi 2004, Agarwal and Pandey 2004) and plant cell membrane (Lüttge 1993) relating to the salinity, relatively little work has been conducted cytochemically on the influence of salinity at cellular level especially, the deposition pattern of H_2O_2. The localization of H_2O_2 would provide the insight to its antioxidant mechanism in each cell organelle under salt stress. In this study, we applied cytochemical method which is based on the reaction of H_2O_2 with $CeCl_3$ to produce electron dense insoluble precipitates of cerium perhydroxides, $Ce(OH)_2OOH$ and $Ce(OH)_3OOH$ (Bestwick *et al*. 1997) in order to examine the accumulation sites of H_2O_2 induced by salinity stress. This ultrastructural technique allows us to observe the precise accumulation site of H_2O_2 under salt stress.

The seeds of rice (*Oryza sativa* L.) salt tolerant cultivar Pokkali, were sterilized with 3 % (v/v) sodium hypochlorite for 15 min and washed thoroughly with distilled water, and seeds were germinated in a Petri dish. Uniformly germinated seeds were selected and transferred to Murashige and Skoog (MS) liquid medium. The seedlings were grown in greenhouse for two weeks and then were supplemented with 100 mM NaCl for 7 d while those without NaCl served as controls.

Received 29 November 2004, *accepted* 11 October 2005.
Abbreviations: CA - sodium cacodylate; EDXA - energy-dispersive X-ray analysis; GA - glutaraldehyde; Mops - 3-(N-morpholino) propanesulfonic acid; MS - Murashige and Skoog; PA - paraformaldehyde; ROS - reactive oxygen species; TEM - transmission electron microscopy.
Acknowledgement: This project was supported by a grant of the Nuclear R&D Program from Ministry of Science and Technology, Korea.
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H_2O_2 was detected by the cerium chloride ($CeCl_3$) method, as described by Bestwick *et al*. (1997). In brief, small pieces (1 mm^3) from leaf sheath, leaf blade, and root of 3-week-old seedlings were excised and incubated in freshly prepared 50 mM Mops [3-(N-morpholino) propanesulfonic acid] buffer, pH 7.2, containing 5 mM $CeCl_3$ for 1 h. Subsequently, the samples were fixed in a mixture of 1.25 % (v/v) glutaraldehyde (GA) and 1.25 % (v/v) paraformaldehyde (PA) in 50 mM sodium cacodylate (CA) buffer, pH 7.2 for 1 h. The samples were washed in CA buffer, post-fixed for 1 h in 1 % (m/v) osmium tetroxide (OsO_4) in sodium CA buffer, dehydrated in a graded acetone series, and embedded in Spurr's resin (Spurr 1969). Ultrathin sections (70 - 90 nm in thickness) were mounted on the uncoated nickel grids (300 mesh) and examined with a transmission electron microscope (TEM) in conjunction with energy-dispersive X-ray analysis (EDXA) (*EM 912*, *Carl Zeiss*, Oberkochen, Germany) at 80 kV without post-staining. To confirm the specificity of $CeCl_3$ staining for H_2O_2, catalase was used to decompose H_2O_2. Samples were incubated for 20 min in 50 mM Mops buffer, pH 7.2, containing 25 mg mm^{-3} bovine liver catalase. They were then transferred to $CeCl_3$ solution and incubated for

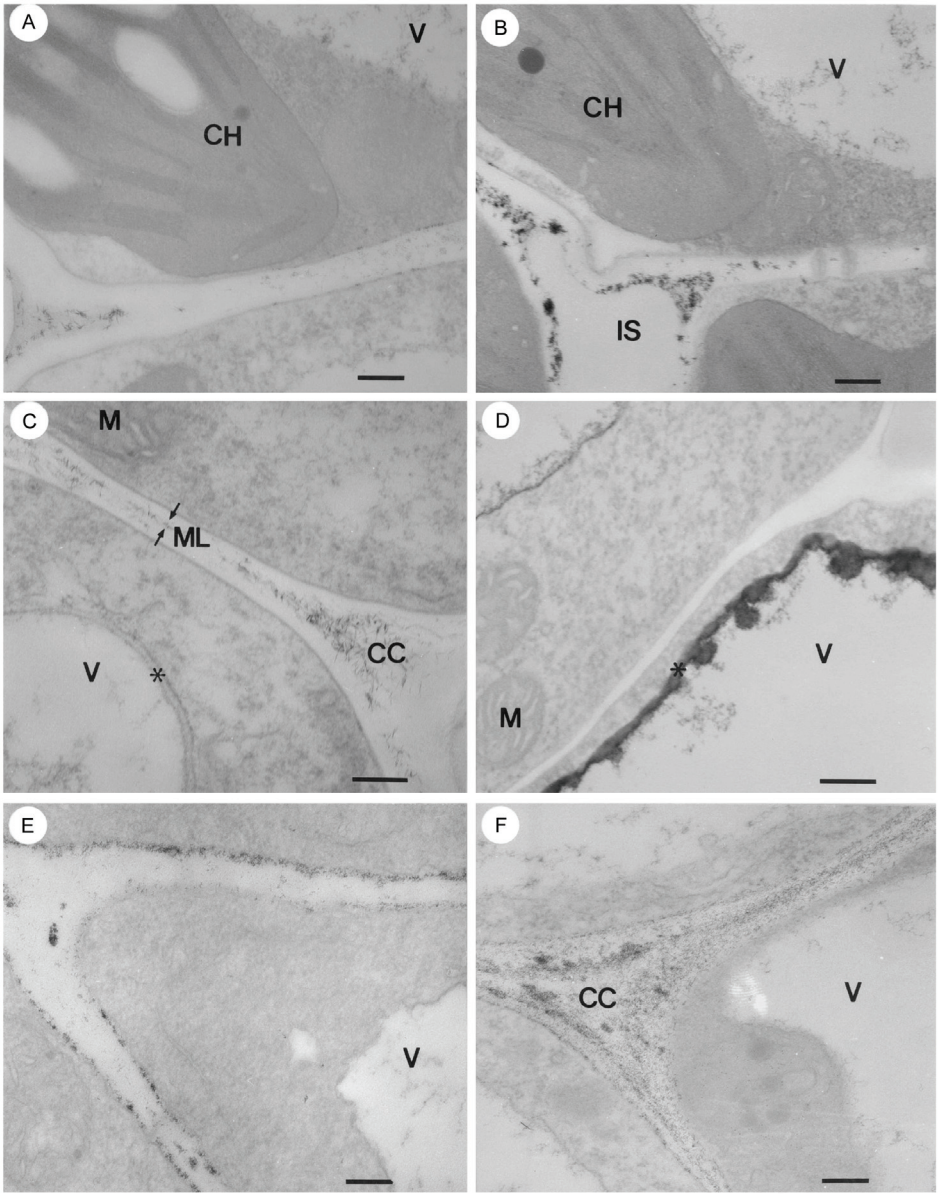

Fig. 1. Visualization of H_2O_2 by cerium perhydroxide deposits in leaf blade (*A*), leaf sheath (*C*), and root (*E*) of rice under normal condition and in leaf blade (*B*), leaf sheath (*D*), and root (*F*) of rice under salt stress. Cerium perhydroxide precipitates are present in the tonoplast surrounding vacuole, in the cell wall, and plasmalemma. CC - cell corner region, CH - chloroplast, IS - intercellular space, M - mitochondria, ML - middle lamella, V - vacuole. *Asterisks* - the region of EDXA analysis. *Bar* = 200 nm.

1 h and processed for TEM as described above. Some samples were fixed in a mixture of 1.25 % (v/v) GA and 1.25 % (v/v) PA in 50 mM CA buffer, pH 7.2 for 1 h without $CeCl_3$ treatment. After postfixation with OsO_4, samples were processed for TEM as mentioned above.

In the control plants, electron dense precipitates of cerium perhydroxides, formed by a reaction of $CeCl_3$ with H_2O_2, were mainly localized within the middle lamella and the corner of cell wall and plasma membrane in leaf blade, leaf sheath and root (Fig. 1*A,C,E*). This result was in good agreement with previous work (Wi *et al*. 2000). Cerious deposits were much more intense in middle lamella than in secondary wall of all tissues. These distribution patterns were also shown in the leaf blade and root (Fig. 1*B*,*F*), but not in the leaf sheath after salt treatment (Fig. 1*D*). There was only little presence of cerium perhydroxide participates in cytoplasm of leaf blade and root. It is not surprising to find labelling of cerium perhydroxide deposits in cell wall, because H_2O_2 is commonly present in primary cell wall and is involved in a wide range of functions, including cell wall extension during plant growth (Fry 1998, Rodriguez *et al*. 2002). In addition, H_2O_2 is necessary for the peroxidase-mediated oxidative polymerization of monolignol to lignins in cell wall (Czaninski *et al*. 1993, Müsel *et al*. 1997, Ros Barceló 1997, Wi *et al*. 2000), although H_2O_2 is accumulated at low concentration under non-stressed conditions.

In the leaf sheath of cv. Pokkali, the electron dense participates of cerium perhydroxides were obviously localized on the tonoplast of bundle sheath together with negligible cerium perhydroxide precipitates on the plasma membrane and cell wall after NaCl treatment (Fig. 1*D*). This phenomenon is similar to the previous result reported by Romero-Puertas *et al*. (2004) who found H_2O_2 accumulation along tonoplast of bundle sheath cell in cadmium-treated pea leaves. According to the result of TEM observation, the high accumulation of H_2O_2 along the tonoplast in leaf sheath under salt stress suggested that the tonoplast may be a major source of H_2O_2. This phenomenon suggested that the production or the deposition of ROS in tonoplast was due to a diminished antioxidant capacity (Romero-Puertas 2004), although scavengers of ROS such as superoxide dismultase, ascorbate peroxidase, and catalase, generally increased their activity more in the salt-tolerant cultivars than in the salt-sensitive cultivars under salt stress (Dionisio-Sese and Tobita 1998, Vaidyananthan *et al*. 2003).

Thorough elemental composition analysis of electron dense deposits in tonoplast was performed by a TEM-EDXA in order to confirm the cerium perhydroxides (Fig. 2). The signal of cerium was not detected on tonoplast of control (Fig. 2*A*). In the NaCl-treated rice (Fig. 2*B*), however, the EDXA spectrum showed the cerium perhydroxide deposition on tonoplast while the Ti signals are attributed to the detector of EDXA. Cerious participates on the tonoplast of leaf sheath of cv. Pokkali after salt treatment were confirmed in the aid of TEM-EDXA. This result clearly demonstrated that the H_2O_2 is localized on the tonoplast of leaf sheath in Pokkali under salt stress.

For the confirmation of the specificity of $CeCl_3$, the staining was examined in the presence of catalase or without $CeCl_3$. All the tissues treated with the catalase or without $CeCl_3$ did not show any accumulation of deposits of cerium perhydroxides in cell walls and membranes, indicating the specificity of cerium chloride staining for the localization of H_2O_2 (data not shown).

In conclusion, even though most of cerium perhydroxides resulting from the reaction of H_2O_2 with $CeCl_3$ were deposited on the plasma membrane and the cell wall in leaf blade and root, they were unusually accumulated on the tonoplast in leaf sheath of Pokkai under 100 mM NaCl. This observation is the first report to show high H_2O_2 accumulation in the tonoplast from leaf sheath under salinity stress.

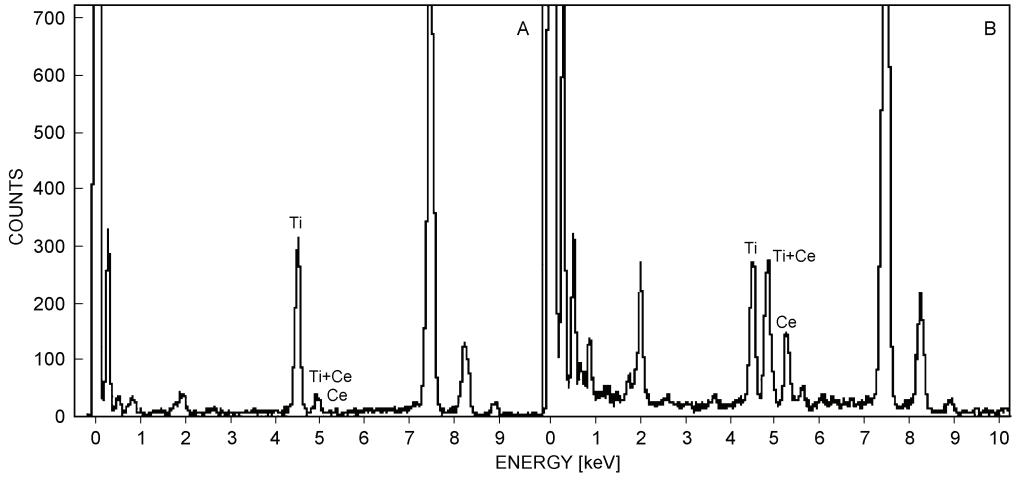

Fig. 2. Spectra of TEM-EDXA obtained from asterisked region of Fig. 1*C,D* (*A* - control, *B* - salt treated sample). Cerious deposits are confirmed in the tonoplast using EDXA. Note the peak of cerium. Titanium derived from EDXA detector. Ce - cerium, Ti - titanium.

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