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CHANGES IN ABSCISIC ACID IMMUNOLOCALIZATION IN HEAT-STRESSED PEPPER SEEDLINGS

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Abstract

The heat stress-responsive abscisic acid (ABA) subcellular distribution in pepper mesophyll and root cap cells was investigated by colloidal gold labeling technique. The results showed that the ABA was localized in the nucleus and the cytoplasm of two cell types in the seedlings under normal temperature, with relatively a higher accumulation in the root cap cells. As the seedlings were transferred to 40°C for heat stress, the ABA levels in both mesophyll and root cap cells increased markedly, especially in the later. With a sustained heat stress, the ultrastructure of mesophyll cell was severely damaged and more ABA accumulated in the nucleus of mesophyll cells; comparably, the root cap cell maintained intact ultrastructurally, and a concomitant drastic increase in ABA in the nucleus of root cap cells was also observed. The above results imply that ABA might be one of the heat stress signaling members in plant cells, whereas the mechanism by which ABA functions during this process remains poorly understood.

Introduction

Abscisic acid (ABA), a phytohormone highly conserved in higher plants, plays multiple roles in plant growth, development and cellular signaling. Emerging evidence also suggested that ABA may function as a signal molecule against environmental stresses (Leung & Giraudat, 1998). It was reported that ABA accumulated quickly and drastically in plant cells in response to flood (Pastor *et al*., 1999), drought van (Rensburg *et al*., 1996), cold (Shinozaki *et al*., 1996) and freeze (Welbaum *et al*., 1997). Additionally, it was also indicated that ABA was involved in the Ca^{2+} signaling pathway (McAinsh *et al*., 1990, Freundl *et al*., 2000).

ABA is synthesized in cap cells or mesophyll cells and then accumulates in plant leaves or seeds (Chanson & Pilet, 1982, Koiwai *et al*., 2004). The endogenous ABA could be transported from roots to leaves and subsequently distributes throughout the whole plant body. At subcellular level, however, the distribution pattern of ABA has not yet been fully elucidated, particularly under stressful conditions. Chloroplast has been proposed to be an ABA trapping compartment and alteration of the ABA compartment has been suggested as a prerequisite for plants to trigger the stress response. Apparently, information about ABA distribution at cellular level under stress condtion will shed further light into the physiological significance of ABA as a stress hormone.

To the best of our knowledge, no data has been reported on the localization of ABA in the plant cells, which are subjected to heat stress. Using immunocolloidal gold labeling technique, in this study, we demonstrated that heat stress could lead to an increase in ABA biosynthesis and alter its subcellular distribution in both mesophyll and root cap cells, implying a role for ABA in the response of plant cell to heat stress.

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Materials and Methods

Plant material: Pepper seeds (*Capsicum annuum* var.grossum Sendt cv.Xingyan No.10) were surface-sterilized in 70% ethanol for 10 min., followed by a few washes in distilled water and then germinated on filter paper soaked with distilled water at 28/24^{o}C (day/night) for 3~4d.The seedlings were grown in plastic tubes containing vermiculite filled with one fourth strength of Hoaglands nutrient solution, pH 6.5.

Heat stress treatment: When the third leaf emerged, the seedlings were transferred to 40^{o}c for heat stress treatment. The heat stress was conducted in the dark and this was done to minimize the amount of starch in the tissue (van Rensburg *et al*., 1993).

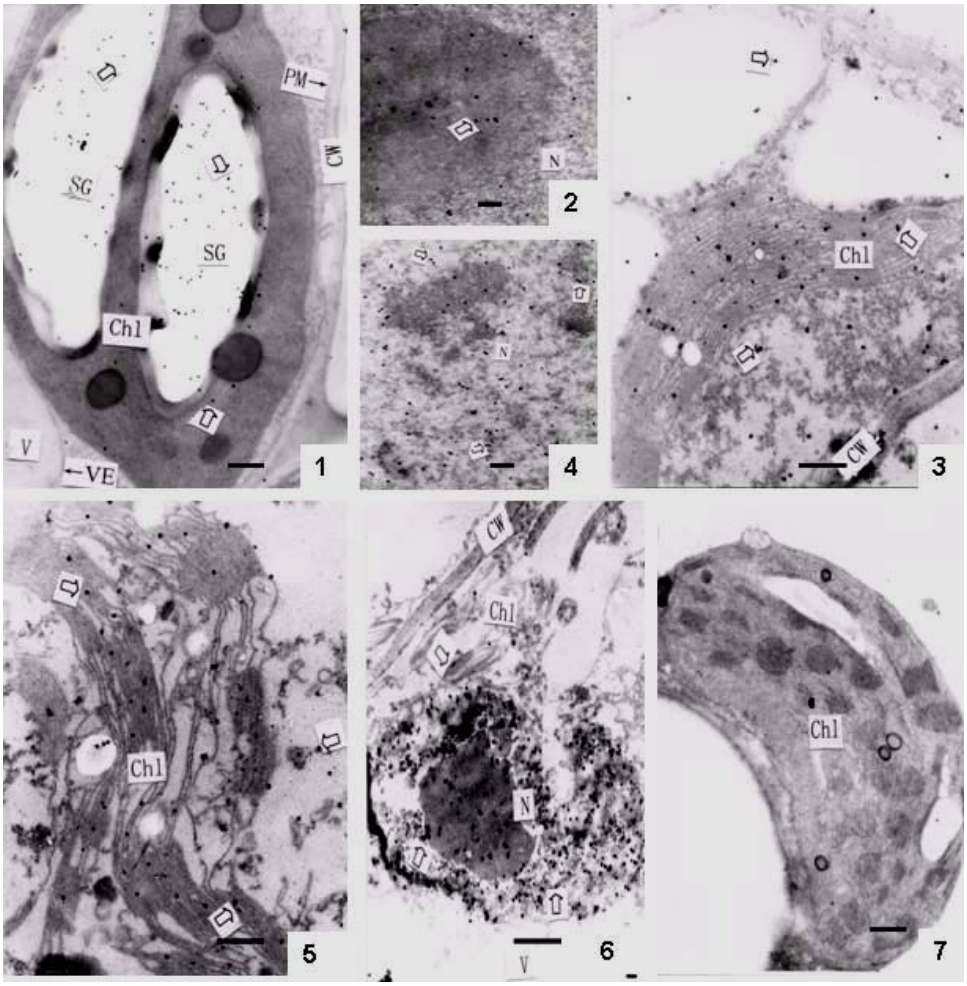
Immunocytochemistry of ABA: Experiment was conducted according to the method of Pastor *et al*., (1999) (Pastor *et al*., 1999) with a little modification. Briefly, pepper leaves were cut into pieces of slices (mm^2) and immediately fixed at reduced pressure in a 2% (w/v) solution of 1-(3-dimethylaminopropyl)-3 ethyl carbodiimide (EDC: dissolved with PBS, 20 mM, pH7.4) for 30 minutes and then in EDC for 2 hours at 4^{o}C and postfixed with 0.5% (w/v) OsO_4 in the same buffer for 40 min. Specimens were subsequently dehydrated in a graded acetone series and embedded in Spurrr′s resin. Ultrathin sections were placed on nickel grids.

For the immunolocalization of ABA, a specific monoclonal antibody against (±) ABA (Purchased from Nanjing Agricultural University, P.R. China) was utilized. Immunolabeling was performed by placing the grid section down, on drops of H_2O_2 (10%) for 10 min. The grids were then transferred to the drops of blocking solution (PBS with 1% bovine serum albumin, BSA) for 20 min. After rinsing in several changes of PBST (PBS containing 0.02% Tween-20, 0.02% NaN_3, pH7.4), the grids were incubated with a drop of the monoclonal anti-ABA antibody, which was diluted with PBST (containing 1% BSA) (1:600) at 30^{o}C for 4h. After washing in several changes of PBST, the grids were then floated on a 1:35 (v/v) dilution of anti-mouse IgG Au (10-15nm) in PBST (containing 1% BSA) at 30^{o}C for 70 min. The grids were rinsed in PBST and distilled water. Controls were as follows: Omission of the primary antibody, incubation with anti-ABA antibodies previously absorbed with an excess of ABA for 24h at 4^{o}C and incubation with pre-immune serum instead of the antibody anti-ABA. Grids were subsequently stained with saturated uranyl acetate solution. Electron micrographs were obtained with a JEM-100CX/II transmission electron microscope at an accelerating voltage of 80 kV.

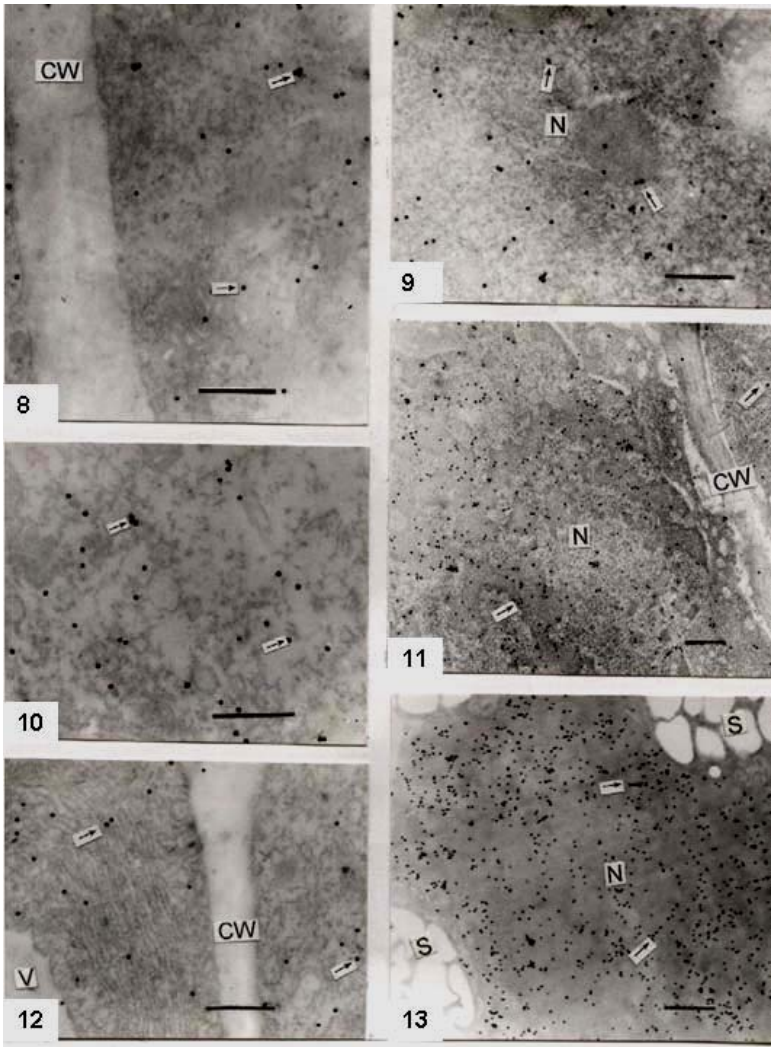
Results

ABA subcellular localization in mesophyll cells: Studies of the intracellular localization of ABA may aid in understanding its physiological functions when cells are under heat stress. Under normal temperature, mesophyll cells contained chloroplasts with well-developed grana and stroma thylakoid. Starch grains were predominantly detected in the dense stroma. The ABA labeling signal was sparse and most of them were distributed in the nucleus and chloroplasts (Figs. 1, 2). When the seedlings were transferred to 40^{o}C for 12 h, the ultrastructure of cells started to alter with the heat time lapsing, such as numerous vesicles emerging, starch grains disappearing gradually and chloroplasts were irregular in shape (Figs. 3, 4); under heat stress for 24h, fragments of chloroplasts membranes were

observed. On the other hand, the density of ABA labelling in the heat stress-imposed cells increased gradually and gold particles were dominantly localized in nucleus and chloroplasts. Furthermore, only a residual of ABA labelling signal was recorded in cytosols and more ABA was concentrated in the dissected nucleus (Figs. 5, 6), suggesting the ABA fluxes within a cell or through adjacent cells, or biosynthesis *de novo* under heat stimulation. No positive reaction for ABA labeling was observed in the control sections with omission of the primary antibody (Fig. 7), indicating the specificity of the antibody against ABA.



Figs. 1-7. Electron-micrographs of ABA localization in pepper mesophyll cells.
1-2. ABA was localized in the cell wall, plasma membrane, grana and stroma thylakoids, starch grains and nucleu. Normal temperature. 3-4. the density of ABA increased in the heat stressed mesophyll cells, especially in the chloroplast and nucleus compartments, and the granular began to loose and swell. 12 h; 5-6. The density of ABA further increased in the nucleus and chloroplasts with a severe heat stress, and the ultrastructure was destroyed. 24 h; 7. Control section. No ABA was observed. Omission of primary antibody.



Figs. 8-13. Electron-micrographs of sections of pepper root cap cells with ABA location.
8-9: Most particles were found in the nucleus and cytoplasm. Normal temperature; 10-11. ABA increased in the cytosol and nucleus after heat stress, but little was found in the cell wall. 12h; 12-13. After sustained heat stress, ABA increased drastically, and most ABA labeling gathered in the nucleus, but no in the starch grains. 24 h.
Chl: Chloroplast S: Starch grain M: Mitochondria Is: Intercellular space V: Vacuole CW: Cell wall. Bar = 1 μm

ABA subcellular localization in root cap cells: It has been demonstrated that root cap cells are the major site of producing endogenous ABA. This fact tempted us to examine the changes of ABA in root cap cells under heat stress. Compared with the mesophyll cells, cap cells held higher density of ABA labeling signal under normal temperature and most signals were concentrated in the nucleus and cytoplasm (Figs. 8, 9). After heat stress for 12 h at 40^{o}C, the ABA levels in the nucleus increased significantly, likely due to the *de novo* synthesis of ABA in root cap cells upon heat stress (Figs. 10, 11). With a sustained heat treatment, however, the ABA labeling signal was detected throughout the cytosol and the amount of ABA reached a higher level (Figs. 12, 13).

Discussion

Observation of ABA subcellular localization in this study showed that ABA is abundantly present in the nucleus, chloroplasts, but little in the vacuoles under normal temperature. The density of ABA in root cap cells is higher than that in mesophyll cells and the nucleus holds a higher ABA density compared with other organelles. These results are consistent with previous results (Hartung & Slovik, 199, Huang *et al*., 1999), suggesting that alkaline compartments such as chloroplast may be prone to accumulate ABA and that root cap cells are the major site of ABA biosynthesis. ABA could be synthesized in several different parts of plants such as isolated leaves, fruits, stems and seeds (Milborrow, 2001) and the ability of different organs to biosynthesize ABA may vary.

At the organ level, previous studies on the ABA signaling events are mainly focussed on the epidermis, thanks to its guard cells that are the targets of ABA action for stomatal closure. It was indicated that epidermis tissue is unable to produce ABA, so that ABA would have to be imported from the mesophyll tissue (Walton, 1980). At an unstressed typical apoplastic pH of 6.5, more ABA will be present in the undissociated lipophilic form (ABAH), which readily diffuses across the plasma membrane into the more alkaline cytoplasmic compartments of mesophyll cell (Hartung & Slovik, 1991). In present study, when the pepper seedlings were heat-stressed at 40^{o}C for different time course, the ABA levels increased significantly in mesophyll cells and there appears the highest ABA density in chloroplasts and nucleus among the mesophyll cell compartments. The heat stress-induced increase in ABA amount in the both organelles may have been caused by biosynthesis *de novo*, just like the case by drought or water stress performed (Pastor *et al*., 1999; Mansfield & McAinsh, 1995, Ren *et al*., 2007), or by the release of ABA from the roots as postulated by other researchers (Zhang & Davies, 1990, Zhang *et al*., 1996). The ABA increase observed in chloroplasts of heat-stressed pepper seedlings could be explained by action of chloroplasts as an anion-trap for weak acids due to photosynthetic stromal alkalization (Zeevart & Creelman, 1988, Hartung, 1981).

Root tips have been regarded as the main sites for ABA biosynthesis and the synthesized ABA will be transported to the target tissues (Chanson & Pilet, 1982, Zhang & Davies, 1990, Zhang *et al*., 1996). Koiwai *et al*., (2004) demonstrated, using immunohistochemical technique, that the guard cells themselves are able to synthesize ABA as well as the root cells. In this text, imposition of heat stress on the pepper seedlings for different intervals, the ABA labeling in root cap cells increased markedly and more ABA was accumulated in the nucleus or cytosol, which is consistent with the conclusion drawn by previous works. However, our results also show that the increase in chloroplast ABA was not followed by a decrease in root cap cell ABA, which implies the increased chloroplast ABA might result from other tissues including guard cells and the root cap cells. Unfortunately, there are no unambiguous evidences defining or excluding the status of mesophyll cells in the process of ABA biosynthesis at diverse levels and hitherto it is difficult to clarify the source of mesophyll cell ABA under heat stress. Anyhow, it couldn't be ruled out that the increased ABA in mesophyll cells upon heat stress might come from the *de novo* biosynthesis within mesophyll cells. The future challenge might be to explore these unresolved issues concerning the track of ABA metabolism under heat stress.

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