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The changes of organelle ultrastructure and Ca^{2+} homeostasis in maize mesophyll cells during the process of drought-induced leaf senescence

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Abstract The changes of cell ultra structure as well as Ca^{2+} homeostasis involved in the drought-induced maize leaf senescence was investigated. Meanwhile, many indicatives of leaf senescence including thiobarbituric acid reactive substance (MDA), electrolyte leakage (EL), and chlorophyll along with soluble proteins were also detected during the process. The Polyethylene glycol6000(PEG6000)-incubated detached leaves showed a slight increase in the MDA content and electrolyte leakage during the first 30 min of our detection, which was corresponded to an unobvious alteration of the cell ultrastructure. Other typical senescence parameters measured in whole leaf exhibited a moderate elevation as well. Thereafter, however, the EL and MDA rose to a large extent, which was correlated with a dramatic damage to the cell ultrastructure with concomitant sharp decrease in the chlorophyll and soluble proteins content. The deposits of calcium antimonite, being an indicator for Ca^{2+} localization, were observed in the vacuoles as well as intercellular spaces in the leaves grown under normal condition. Nevertheless, after PEG treatment, it was revealed a distinct increment of Ca^{2+} in the cytoplasm as well as chloroplasts and nuclei. Moreover, with long-lasting treatment of PEG to the detached leaves, the concentration of Ca^{2+} as described above showed a continuous increment which was consist with the remarked alteration of physiological parameters and severe damage to the ultrastructure of cells, all of which indicated the leaf senescence. Such drought-induced leaf senescence might result from a loss of the cell's capability to extrude Ca^{2+}. All above findings give us a good insight into the important role of Ca^{2+} homeostasis in the process of leaf senescence accelerated by the drought stress.

Keywords: Ca^{2+} homeostasis, drought, leaf senescence, maize, organelle ultrastructure, signal transduction

INTRODUCTION

Leaf senescence is the final stage in leaf development, and better understanding senescence is important not only for purely scientific reasons, but also for practical purposes. Leaf senescence is not simply the aging-dependant passive death of a leaf, but is a tightly organized and controlled process during which cell components are degraded in a coordinated fashion. When nutrients have been relocated to other parts of the plant body, the cell finally dies (Gan and Amasino, 1997; Noode'n et al. 1997; Shao et al. 2008a; Shao et al. 2008b; Shao et al. 2008c; Shao et al. 2009).

Conspicuous visual symptoms of leaf senescence are the loss of chlorophyll pigments (yellowing), desiccation, and eventual abscission. Cellular and molecular events contributing to these visual

symptoms involve chloroplast disintegration, a decline in photosynthesis, and the loss of proteins and nucleic acids (Smart, 1994; Buchanan-Wollaston, 1997; Chandlee, 2001). Additional internal symptoms of senescence are a decreased ability to accumulate proteins and nucleic acids due to enhanced degradation and/or reduced synthesis (Smart, 1994; Buchanan-Wollaston, 1997). The catabolism of chlorophylls, lipids, proteins as well as nucleic acids allows large amounts of nutrients released and mobilized subsequently to growing leaves, developing seed, or storage tissues (Buchanan-Wollaston, 1997; Quirino et al. 2000), since they can move up or down readily within the phloem, such as nitrogen, potassium, phosphorus and magnesium (macronutrients, except Ca and S).

Although senescence occurs in an age-dependant manner in many species (Noode'n, 1988), its initiation and progression can be modulated by a variety of environmental factors such as temperature, mineral deficiency, and drought conditions, as well as by internal factors such as plant growth regulators (Grbić and Bleecker, 1995; Nam, 1997; Weaver et al. 1997; Dai et al. 1999). It has proved that drought, darkness, leaf detachment, and the hormones abscisic acid (ABA) and ethylene induce leaf yellowing, which is an good indicator of leaf senescence, whereas, to different extent.

Calcium (Ca^{2+}) plays a crucial role in plant membrane stability, cell wall stabilization, and cell integrity (Hirschi, 2004). On other hand, Ca^{2+} has been proven a sensor to multiple and variable environmental signals, which means stimulus-response coupling for these signals can be widely mediated (at least in the early steps) by modulation of cytosolic free Ca^{2+} (Sanders et al. 1999; Shao et al. 2008a; Shao et al. 2008c; Shao et al. 2009), which in turn is modulated by calcium-calmodulin. It has been well evident various environmental conditions and hormone signal molecules as wind, illumination, low temperatures, salt, alkali, gibberellins, ABA can trigger the alteration of cytosolic Ca^{2+} concentrations, which leads to plants' response (McCue and Hanson, 1990; Wood et al. 2000). However, it also has been pointed out that only if the concentration and distribution of cytosolic Ca^{2+} alters in a proper scope (100-200 nM), can Ca^{2+} take an active role in adaptation of plant to environment stimuli. If it is beyond the proper range, it could disturb or break the normal structure and function of cells (Bush, 1993), thus facilitate the senescence.

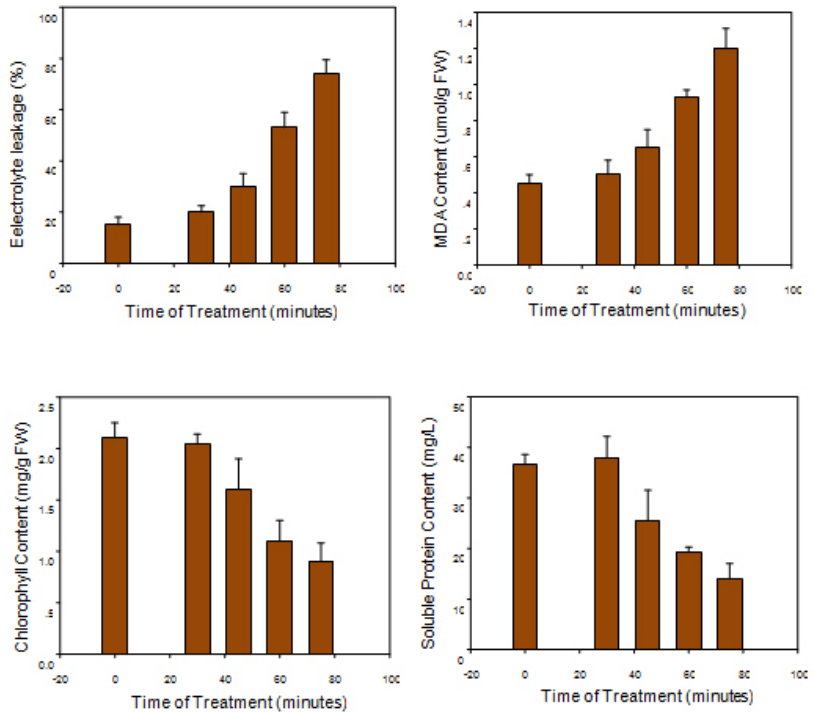

Fig. 1 Impacts of drought stress on Ca^{2+} homeostasis and ultrastructure in mesophyll cells.

Drought is a common stress condition that adversely affect plant growth and crop production. It can make plants senesce early, which leads to the failure in the yield of crops. It has been asserted that many senescence-associated genes was isolated from cowpea subjected to the drought stress (Muchero et al. 2008), which provides additional evidence for the induction of leaf senescence by drought (Shao et al. 2008a; Shao et al. 2008c; Shao et al. 2009).

All above facts triggered our interest to further investigate the impact of Ca^{2+} homeostasis on maize's response to drought stimuli. Our additional focus is on the drought-induced leaf senescence in maize as well as the relationship between Ca^{2+} and the induction of leaf senescence by drought stress. In this study, we examined physiological parameters to better understand the influence of PEG that imitated the effect of drought on maize detached leaves. Meanwhile, we investigated the alterations in ultra structure of cells as well as the distribution along with the concentration of the Ca^{2+} during the course of the leaf senescence, which made us get the more information about the possible impact of Ca^{2+} homeostasis on the delayed senescence in maize.

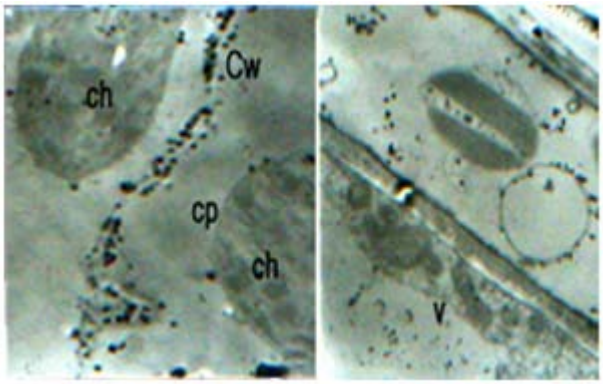

Fig. 2 The cellular Ca^{2+} distribution in vacuoles, intercellular spaces and chloroplast in leaf cells of maize grown under normal condition.

MATERIALS AND METHODS

The fully-nourished seeds of one maize hybrid "zhengdan958" (*Zea mays* L.) were selected, sterilized by 0.1% $HgCl_2$ for 10 min and repeatedly washed by water. After being soaked in the water for 24 hrs, the seeds were cultured in an incubator for germination with a constant temperature of 28ºC. When the bud length reached to 1 cm, the seeds were planted into vermiculite pots. While the first true leaves were entirely extended, the seedlings were sampled.

The leaves which were morphologically similar were detached and dipped in the Polyethylene glycol6000 for 30, 45, 60, 75 min respectively. Thereafter, the leaves were collected for measurements of physiological standards and cytochemical localization of Ca^{2+}.

Extraction and determination of lipid peroxidation product

TBA-reactive substance MDA representing lipid peroxidation products were extracted as described previously by homogenization of 0.5 g of tissue in 5 mL of solution containing 20% TCA and 1.5 mM EDTA (Meir et al. 1992). MDA was assayed using the TBA test (Kosugi and Kikugawa, 1985), as modified by Meir et al. (1992). One milliliter of 0.67% TBA was added to 3 mL aliquots of the supernatant and the solution was incubated at 100ºC for 1 hr. The solution was then cooled and centrifuged for 10 min at 8000 rpm.

The volume of the resultant supernatant was made up to 10 mL with distilled water, and the A455 and A532 of the coloured reaction product were determined for aldehydes and MDA, respectively. Three replicates were used for each assay.

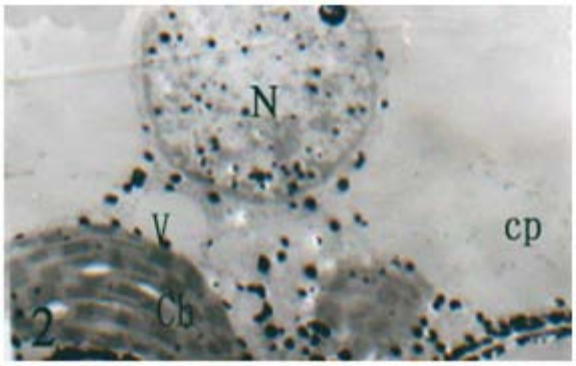

Fig. 3 The cellular Ca^{2+} distribution in inner side of plasma membrane, intercellular space and vacuole of maize leaf cells after treatment with PEG for 30 min.

Determination of electrolyte leakage

Drought injury was assessed as electrolyte leakage (EL). It was measured by using leaf segments (5 mm size) for each treatment. Samples were washed with deionised water to remove surface adhered electrolytes. These were placed in closed vials containing 10 mL of deionised water and incubated at 25ºC on a rotary shaker for 24 hrs and subsequently electrical conductivity of the solution (L1) was determined. Samples were then autoclaved at 120ºC for 20 min and the final electrical conductivity (L2) was obtained after equilibration at 25ºC. The EL was defined as follows: EL(%) = (L1/L2) x 100.

Chlorophyll content determination

Chlorophyll (Chl) content was determined as described before (He et al. 2002). For the determination of the Chl content, 0.25 g of fresh leaves were placed in a 100 cm^3 test tube; 10-15 cm^3 pure methanol was added and homogenized with a polytron. The homogenate was then filtered and filled up to 100 cm^3 with pure methanol. The Chl concentration in the supernatant was spectrophotometrically determined by measuring the absorbances at 652 and 665 nm for Chla and Chlb, respectively, and calculated according to Porra et al. (1989).

Soluble protein determination

The soluble protein content in maize leaves was analyzed as below: Frozen leaves were ground in liquid nitrogen to a fine powder and suspended in a buffer containing 100 mMNaH_2PO_4/Na_2HPO_4 (pH 7.0), 1 mM phenylmethylsulphonylfluoride, 1%(v/v) β-mercaptoethanol, and then centrifuged for 5 min at 9800 g. The soluble protein concentration in the supernatant was determined using BSA as a standard.

Determination of Ca^{2+} localization

Ca^{2+} localization in maize mesophyll cells was conducted according to the method of Tian. Disks of the leaves were fixed in 2% glutaraldehyde in 0.1 M KH_2PO_4 buffer (pH 7.8) containing 1% $K_2H_2Sb_2O_7 \cdot 4H_2O$ for 4 hrs at room temperature, and washed five times with the same buffer with no $K_2H_2Sb_2O_7 \cdot 4H_2O$ for 20 min of each washing, then post-fixed in 1% OsO_4 in 0.1 M KH_2PO_4 buffer containing 1% $K_2H_2Sb_2O_7 \cdot 4H_2O$ at 4ºC for 16 hrs. Prior work has indicated that temperature has no significant impact on labeling. Disks of the leaves were then washed in four changes of 0.1 M KH_2PO_4 buffer without antimonite, dehydrated in a graded acetone series and embedded in Epon812 resin. Disks were stained with uranyl acetate and observed with a H7500 transmission electron microscope (Hitachi Company, Japanese) to verify the real localization of the Ca^{2+}. Additional control was carried out as followed: The photographed slices were placed into solution of ethylene glycol-bis(2-minoethylether)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA, a kind of Ca^{2+} chelator) for its chelating with Ca^{2+},

and then photographed secondly when the dense electronic particles disappeared, thus determining the previously-acquired situation of Ca^{2+} distribution.

RESULTS

Determination of drought-triggered senescence in maize leaves

The effect of drought stress on the maize leaves was detected by incubating the detached maize leaves in the PEG. Meanwhile, by adopting senescence indicators such as Chl and soluble proteins loss and EL along with the accumulation of MDA, the rate of leaf senescence were determined periodically during drought-accelerated senescence of maize leaves.

It was observed that drought stimulus by the PEG-incubation caused increment of EL and MDA in leaves of the maize. The EL and MDA rise slightly during the first 30 min of our observation, while the EL as well as MDA elevated sharply during the subsequent detection and remained a higher increment until the last phase of our investigation (Figure 1).

The chlorophyll level decreased to a less extent at the first 30 min compared with the subsequent process (Figure 1). It showed particularly marked decrease in chlorophyll content during the second stage, and the decrement continued to the 75 min of PEG treatment, which was the last stage of drought stimuli.

The compatible solute such as protein was found to accumulate substantially at the first stage. Nevertheless, the content of these solutes declined from the second stage, and kept the considerate decline all the process, which was corresponded to the significant loss of chlorophyll.

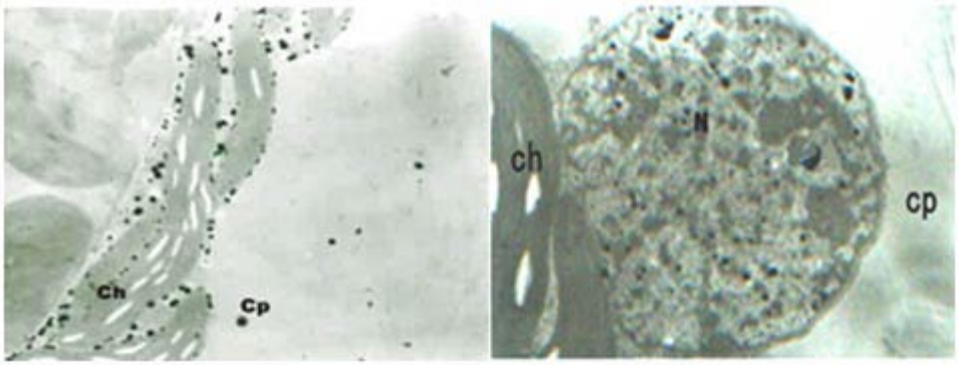

Fig. 4 The cellular Ca^{2+} distribution in chloroplasts as well as nucleus of maize leaf cells after treatment with PEG for 45 min.

It was observed in the detached leaves which were not treated with PEG, the deposits of calcium antimonate being the indicator for Ca^{2+} localization mainly exhibited in the vacuoles as well as intercellular spaces. Meanwhile, the cellular Ca^{2+} distribution showed occasionally in chloroplasts, nucleus and Golgi apparatuses. Whereas, in this detection, no Ca^{2+} localization was found in endoplasmic reticulum (ER) which also represents a commonly-considered cellular Ca^{2+} sink as others reported (Figure 2).

After being soaked in the PEG for 30 sec, it was detected the significant changes of Ca^{2+} distribution. The level of Ca^{2+} increased significantly in cytoplasm, but decreased in vacuoles and intercellular spaces respectively. The Ca^{2+} concentration in chloroplasts and nucleus also showed increment, however, to a less extent as compared with the cytoplasm (Figure 3). At this stage, the ultra structure of cells showed no obvious changes. Whereas, instead of the intact vacuole occupying nearly all the intracellular space, more tiny vacuoles appeared (Figure 3).

With the longer time the leaves were treated, the Ca^{2+} concentration in chloroplasts and nucleus showed continuous increment with concomitant same pattern of alterations in cytoplasmic Ca^{2+} amounts, all of which were correlated with considerate change in the ultrastructure of cells in the PEG-incubation detached leaves. On comparison with the first 30 min, during which just the presence of multivesicular bodies was investigated, it showed nuclear chromatin condensation along with extensive degradation and disorganization of the grana stacks in the chloroplast during subsequent detection. At the last stage, plasma membrane and chloroplasts suffered from the damage severely, indicating ultrastructure of cells was destroyed (Figure 4, Figure 5 and Figure 6).

After EGTA treatment, the precipitates in the slice were eliminated. The original sectors for Ca^{2+} distribution such as intercellular spaces, chloroplasts, vacuoles, and plasma membranes presented transparent electron areas similar as the original precipitate structures, demonstrating a real reflection of Ca^{2+} localization in the slice.

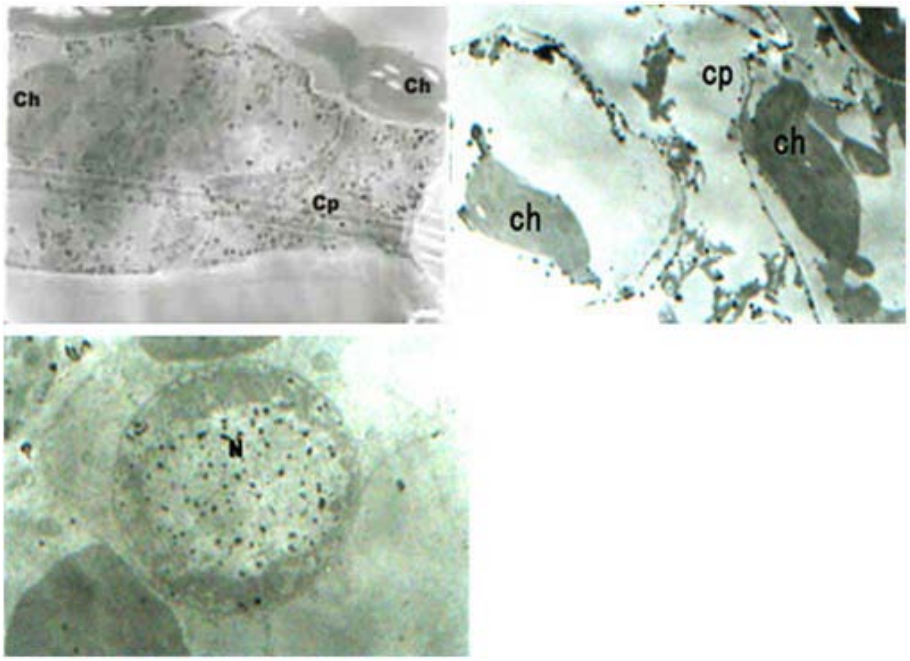

Fig. 5 The cellular Ca^{2+} distribution in leaf cells of maize treated with PEG for 60 min.

DISCUSSION

As our results revealed, after being treated with PEG which initiated the drought stress on the detached leaves, the localization of Ca^{2+} changed remarkably. At normal condition, due to the large amount of Ca^{2+} showing in vacuoles and intercellular spaces, Ca^{2+} level in cytoplasm is considerably low. However, the PEG treatment caused a prominent elevation in cytosolic Ca^{2+} with concomitant sharp decline of Ca^{2+} content in vacuoles and intercellular spaces. It has been concluded one early response to low temperature, drought, and salinity stress in plant cells is a transient increase in cytosolic Ca^{2+}, derived from either influx from the apoplastic space or release from internal stores (Sanders et al. 1999; Knight, 2000). Vacuole H+-ATPase plays important roles in transporting Ca^{2+}, but concrete mechanisms remain unknown (Knight, 2000; Liu et al. 2010). Our experiment results seemed consistent with these conclusions, and also demonstrated the increasing level of Ca^{2+} in cytoplasm induced by the drought stress come from vacuoles as well as the intercellular spaces. In recent years, more attention has been paid to identity the contribution of Ca^{2+} in diverse compartmentalization to cytoplasmic Ca^{2+} responding to differential signals (Blume et al. 2000; Knight, 2000). On the basis of our experiment findings, we suggested under drought stress both intercellular and intracellular organelles contributed to the elevated Ca^{2+} content in intracellular spaces.

The modulation of cytosolic free Ca^{2+} can mediate plants' response to various stimulus, which results in coordination of plants' growth and development (Sanders et al. 2002; Shao et al. 2008a; Shao et al. 2008b; Shao et al. 2008c; Shao et al. 2009). In addition, it has been confirmed that cellular Ca^{2+} changes precede the activation of ABA-, cold-, and osmotic stress-inducible genes (Shinozaki and Yamaguchi-Shinozaki, 2000; Knight and Knight, 2001). For instance, several cold-regulated genes are known to be expressed in response to Ca^{2+} (Monroy and Dhindsa, 1995; Henriksson and Trewavas, 2003). Exogenous application of Ca^{2+} to plants grown at normal growth temperatures has been found to induce cold tolerance (Monroy and Dhindsa, 1995; Nayyar and Kaushal, 2002). In our present study, we found the relatively slight increment in the electrolyte leakage as well as the MDA content at the early stage of PEG treatment, which indicated the appearance of increased Ca^{2+} in cytoplasm might impart the cells enhanced tolerance to the drought stress. Additional proof is the accumulation of soluble proteins, which are thought to play a role in protection of cells from stress injury as osmoprotectants. Therefore, it was speculated that at this stage, drought signal induced plants' response and adaptive to this environmental change through the regulation of Ca^{2+} in localization and distribution. Moreover, there existed other reports which might support our hypothesis (Minorsky, 1989; Poovaiah, 1993).

Nonetheless, at latter stages, with the long term treatment by PEG which imitated lasting drought stress on the detached leaves, dramatic damage to the ultra structure of cells was detectable, including the strong morphological changes in the chloroplasts and nucleus. At last, it was seen that some cells and chloroplasts designated. Corresponding to these morphological alterations was the sharp increase in the EL, MDA concentrations associated with the reduced levels of chlorophyll, soluble proteins which manifested the senescence of the leaves. It has been found by other researchers that leaf senescence caused lipid peroxidation which led to the increment in EL and MDA (He and Jing, 1999; Lim and Nam, 2005). Moreover, the subsequent degradation of chlorophyll and protein is the main biochemical event that occurs in senescent leaves as well. As all above demonstrated, the possibility continuous drought stress resulted in the earlier senescence of maize leaves seemed not unlikely.

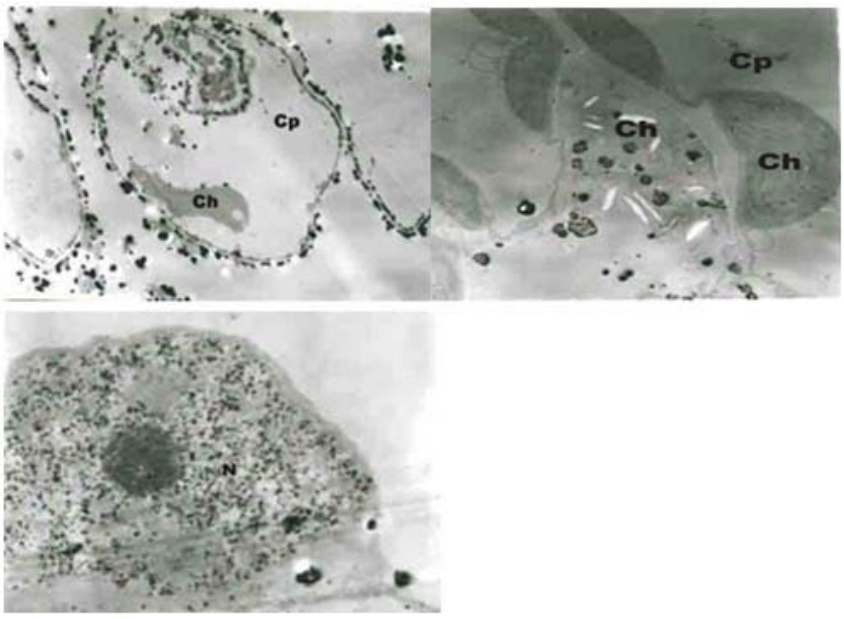

Fig. 6 The cellular Ca^{2+} distribution in leaf cells of maize treated with PEG for 75 min.

In this study, we also investigated the impact of Ca^{2+} homeostasis on the drought-triggered leaf senescence in maize. It proved the free cytosolic Ca^{2+} level is critically important for the control of many essential cellular responses, thus it has to be strictly regulated to gain an accurate function of the cell. Such a specific regulation of many different events has to be carried out by not only modulating the concentration, but also in a spatial and temporal mode (Trewavas, 1999). Cellular Ca^{2+} homeostasis is normally maintained at submicromolar levels by an ensemble of membrane associated

Ca^{2+} transport pumps, secondary transporters, and ion channels (Hepler and Wayne, 1985; Bush, 1995). Stimulus-induced changes in Ca^{2+} concentration may be transient, sustained, or oscillatory, and the time required for full response varies from a few seconds to severa 1 hr (Bush, 1995). However, following the calcium release, efflux systems restore cytoplasm Ca^{2+} concentration to resting levels, thereby terminating a Ca^{2+} signal (Dürr et al. 1998; Shao et al. 2008a; Shao et al. 2008b; Shao et al. 2009).

If the cells lack the capability to extrude the Ca^{2+} and thus cause excess content of Ca^{2+} which is beyond the resting level of Ca^{2+}, the normal structure and function of cells could be disturbed or broken. If the amount rises too high, the energetic metabolism of the formation from ADP to ATP could be destroyed, leading to cells poisoned (Bush 1993; Shao et al. 2008a; Shao et al. 2008b; Shao et al. 2008c).

Further evidence for the involvement of Ca^{2+} homeostasis in the processes resulting in senescence was also deciphered for leaves of cowpea (Savithramma and Swamy, 1989), oat (Dreier, 1990), rice (Chou and Kao, 1992), corn (Huang and Kao, 1992a; Huang and Kao, 1992b). In our study, it also occurred senescence-related elevation of Ca^{2+} concentration in cytoplasm as well as cellular organelles including chloroplasts and nucleus. In particular, there appeared excess amount of Ca^{2+} in nucleus at last stage, which made us take into consideration the Ca^{2+} located in this organelle. It has been well known that compartmentalization takes a central role in Ca^{2+}-related signal transduction events. Measurements of cellular Ca^{2+} have shown that plant cells avoid potentially toxic effects of Ca^{2+} by maintaining levels in both the cytosol and nucleus that are 3-4 orders of magnitude lower than the levels in other cellular compartments. Therefore, it was hypothesized the considerately large amount of Ca^{2+} showcased in the nucleus might take part in the acceleration of the leaf senescence.

During all the process when maize leaves confronted drought stress, not only the localization and concentration of Ca^{2+} but the ultra structure of cells experienced dramatic alteration. It was observed non-treated cells appeared one central vacuole as well as nearly round or slightly ovoid-shaped chloroplasts. Microscopic observations revealed that PEG treatment for 30 sec resulted in a slight alteration in the appearance of intracellular organelles. Slender chloroplasts along with several segmented vacuoles showed in the PEG treated cells. When treated by PEG to 60 min, drought injury-associated ultra structural changes such as formation of a particulate domains and fracture-jump lesions were frequently detected in the plasma membrane as well as the intracellular membrane due to the severe dehydration caused by the PEG, which indicated the drought stress has cause irreversible damage to these cells. During the detection, it seemed the chloroplasts suffered from more prominent damage, which made us think of the possibility that chloroplasts took participate in the induction of leaf senescence. At the whole-plant level, the effect of stress is usually perceived as a decrease in photosynthesis, which has adverse impact on the plants' developmental stage and thus causes the earlier ingoing of leaf senescence. Since the chloroplast is the main place where photosynthesis takes place, it was considered the limitation of photosynthesis involved in the induction of leaf senescence was attributed to the injury of chloroplasts.

In contrast to other researches which concentrate on the participation of Ca^{2+} in sensing and transmitting the various stresses signals, our focus in this study is on the mechanism by which Ca^{2+} homeostasis is involved in the induction of leaf senescence by drought stress. In conclusion, we suggest drought stress in short term is able to elevate the cytosolic Ca^{2+} content in proper scope, which may contribute to the enhancement of plants' tolerance to drought. Whereas, the long-lasting drought stress leads to the loss of cells ability to efflux the Ca^{2+}, followed by the incapability of cells to maintain the Ca^{2+}, which finally result in the early senescence of leaves. On the other hand, the cellular organelles including chloroplasts and nucleus take part in regulating the cytoplasmic Ca^{2+}, whereas, the excess Ca^{2+} concentration along with the prominent damage shown in these two critical organelles might accelerate the leaf senescence.

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