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The dynamic changing of Ca^{2+} cellular localization in maize leaflets under drought stress

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Abstract

Maize cultivar zhengdan958 was selected as materials. The sub-cellular distribution of soluble calcium at different phases was shown by the potassium-pyroantinonate-precipitation method and transmission electron microscopy. The results showed that the deposits of calcium antimonate as the indicator for Ca^{2+} localization were mainly concentrated within the vacuoles and intercellular spaces without PEG treatment. Firstly, when the leaf was treated with PEG, the Ca^{2+} level increased remarkably in the cytoplasm, but considerably decreased in vacuoles and intercellular gaps. Meanwhile, the level of Ca^{2+} also increased in chloroplast and nucleus. When the treatment continued, the level of Ca^{2+} in chloroplasts and nucleus continued to increase and some cells and chloroplasts finally disintegrated, showing that there is a relationship between the distribution of Ca^{2+} and the super-microstructure of cells. Ca^{2+} plays a role in the plant drought resistance. The changes of cytosolic Ca^{2+} localization in cells treated by ABA, EGTA, Verapamil and TFP were investigated too. The increase of cytosolic calcium induced by ABA was mainly caused by calcium influx. Calmodulin participated in ABA signal transduction, which was indicated by the variation of cytosolic Ca^{2+}/CaM concentration change induced by ABA. The above results provided a direct evidence for calcium ion as an important signal at the experimental cellular level. ***To cite this article: Y.Y. Ma et al., C. R. Biologies 332 (2009).***

Keywords: Osmotic stress; Maize; Ca^{2+} localization; Cell super-microstructure; Biomembrane; Signal

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1. Introduction

As an element greatly required for plant physiological and molecular activities, Ca^{2+} plays important roles in regulating plant metabolism, growth and development. Moreover, Ca^{2+} also participates in many physiological and biochemical reactions in plants as a common second messenger through coupling both cellular and intercellular signal transduction networks. Growing evidence shows that cytosolic Ca^{2+} concentrations can be induced to change by various environmental conditions and hormone signal molecules, such as wind, illumination, low temperatures, salt, alkali, gibberellins, ABA (abscisic acid). These changes of cytosolic Ca^{2+} concentrations occur before the physiological activities of the plants [1–15]. Therefore, it is speculated that the change of Ca^{2+} concentration and distribution in plant cells induced by the plant itself may be considered as the plant's responses and adaptations to the ever-changing outside environment. The distribution and translocation of cytosolic free Ca^{2+} serve as fundamental basis for the formation of Ca^{2+} signals. At present, the distribution of cytosolic Ca^{2+} and the change of plant cell's super-microstructures under cold stresses are investigated in depth while the distribution of cytosolic Ca^{2+} and its sub-cellular localization under drought stresses are less studied [16–28].

It has been demonstrated by more research that the addition of calcium to leaves could enhance water conservation capability of both plant leaves and their cellular membranes, thus effectively ameliorating plant water conditions under drought stress [29–41]. It is also considered that Ca^{2+} can alter the hydration degrees of membrane, and improve the cohesion of cell walls, thus enhancing the viscosity of protoplasm and cells' capacity of dehydration resistance. In addition, Ca^{2+} can improve the hydrophobicity of cellular membrane while lowering its permeability through connecting to the phosphates and carboxyl of phosphatides and proteins in cellular membranes, thus strengthening the stability of membranes, which shows that Ca^{2+} can stabilize plant cells through its direct effects as a structural basis for plant drought-resistance [42–59]. The report concerning the change of cellular super-microstructures merely focused on nucleus and chloroplast. In this paper, the change of cytosolic Ca^{2+} concentrations and cellular super-microstructures of maize at different drought stress stages were researched in detail, providing theoretical basis for the investigation of the Ca^{2+} roles in plant drought-resistance. In plant cells, it was already reported that ABA-induced cytosolic Ca^{2+} concentration increase participated in stomatal closure [16–24], while the temporal and spatial specificities of ABA-induced changes of cytosolic Ca^{2+} distribution and concentration are rarely reported. Drought is a major stress to agricultural production. Plants synthesize ABA in response to drought, triggering a signaling cascade in guard cells that results in stomatal closure, thus reducing water loss that may influence water use efficiency in plants. In addition, drought is also related to salt stress, cold stress, high temperature stress, acid stress, alkaline stress, pathological reactions, senescence, growth, development, cell circle, UV-B damage, wounding, embryogenesis, flowering, signal transduction and so on [25–47,60–73]. In this report, the changes of cytosolic Ca^{2+} distribution and concentration are traced and measured through the application of transmission electron microscope (TEM) technique under drought stress, attempting to further discover the role of Ca^{2+}/CaM in the drought signal transduction and the cause of ABA-induced cytosolic Ca^{2+} concentration increase.

2. Materials and methods

The tested material in this experiment is the hybridized maize (*Zea mays* L.) cultivar called "zhengdan958". According to [48–51], the fully-nourished seeds were selected, sterilized by 0.1% $HgCl_2$ for 10 min and repeatedly washed by water. 24 h after seed soaking, the seeds were cultured in an incubator for germination with a constant temperature of 28 °C. When the bud length reached 1 cm, the seeds were planted into vermiculite pots. When the first true leaf was entirely extended, the seedlings were sampled. The sample leaves were quickly soaked into PEG-6000 (−0.85 MPa) for stress treatments of 30, 45, 60, and 75 min, respectively. After these treatments, cytochemical localization was implemented in the cells of sample leaves. The sample leaves were then collected for the following treatments: (1) treated by the direct addition of 50 μM ABA; (2) soaked into 2 mM EGTA solution (supplemented with 10 mM MES, 50 mM KC1, pH 6.1) for 30 min for illumination avoidance and then treated by 50 μM ABA; (3) soaked into 100 μM Verapamil solution (supplemented with 10 mM MES, 50 mM KC1, pH 6.1) for 30 min for illumination avoidance and then treated by 50 μM ABA; (4) soaked into 80 μM TFP solution (supplemented with 10 mM MES, 50 mM KC1, pH 6.1) for 30 min for illumination avoidance and then treated by 50 μM ABA. The cytochemical localization of Ca^{2+} was implemented as following: 2% potassium pyroantinonate solution (represented by "A solution" in the following context, pH 7.6) was formulated with

potassium phosphate. A solution should be formulated as a fixed solution containing 3% glutaraldehyde. The sample leaves were treated by such reagent with a size of 1 mm × 0.3 mm × 10 mm and fixed overnight. After overnight, theses leaves were washed by A solution for 3 times with an interval of 0.5 h for each time. Then, theses samples were fixed again by osmic acid formulated with A solution for 2 h, washed 3 times by double distilled water with an interval of 0.5 h for each time, and then washed twice by double distilled water (pH 10) with an interval of 0.5 h for each time. Next, the samples were placed into ethanol with various concentration gradients, and dehydrated, embedded, ultramicrocut, and dyed with uranium acetate, respectively. After the above operations, the approximate configuration and dense electronic particles of these leaves can be observed under transmission electron microscope (TEM), thus further inferring the location of Ca^{2+}. The Ca^{2+} cellular localization was determined as follows: the photographed slices were placed into EGTA (a kind of Ca^{2+} chelators) for their chelating with Ca^{2+}, the above slices were photographed when the dense electronic particles disappeared, and then the Ca^{2+} cellular distribution was determined.

3. Results

It can be concluded from the results of TEM observation that calcium pyroantinonate precipitate particles created from cellular Ca^{2+} localization appeared in large quantity in vacuoles, and occurred occasionally in chloroplasts, nucleus and Golgi apparatus. It was reported that Ca^{2+} distributed in endoplasmic reticulum (ER) which also represents a commonly-considered cellular Ca^{2+} sink with large quantity, while such large amounts of Ca^{2+} distribution was not found in ER in this experiment. Plant cell wall represents another kind of cellular Ca^{2+} sink, and in this experiment, Ca^{2+} existence was found in cell walls. In addition, large amounts of calcium pyroantinonate precipitate particles were also observed in the intercellular gaps outside the cells (CK).

In Fig. 1.1, it can be observed that 30 min after PEG stress, the free Ca^{2+} concentration in cytoplasm and nucleus considerably increased. The large amounts of Ca^{2+} precipitate particles formed in the internal sides of the plasma membrane represented remarkable marks for such cellular Ca^{2+} concentration elevation. These particles were relatively orderly arranged and almost formed a particle circle in the internal sides of the plasma membrane. In vacuoles, Ca^{2+} was localized in the outside of vacuolar membrane. After PEG stress, vacuolar Ca^{2+} concentration significantly decreased (Fig. 1.1a), and large amounts of Ca^{2+} distribution occurred in the outside of chloroplast capsules (Fig. 1.1a). Contrary to the apparent change in cellular Ca^{2+} concentration, no remarkable change in cellular super-microstructure was observed (Fig. 1.1b). As shown in Fig. 1.2a, 45 min after PEG stress, the cells transformed, but such cell transformation was not significant. Relatively larger Ca^{2+} precipitates can be observed in the outside of cell walls, and relatively huge amounts of Ca^{2+} precipitate particles can also be found in cytoplasm. Compared with those under 30 min PEG stress treatment, the precipitates under this treatment were disorderly and unevenly distributed, and considerably concentrated in certain parts of plant cells. Some chloroplasts expanded and became round with their enlarged internal stromal spaces and expanded granular thylakoid, while the morphological change occurred in some other chloroplasts (Fig. 1.2a). With the continuous increase of Ca^{2+} concentration in the nucleuses and the appearance of larger black particles, the heterochromatin in the cells conglomerated (Fig. 1.2b). With cell transformation, the cytosolic Ca^{2+} concentration enhanced significantly, while the Ca^{2+} particles had irregularity in their configurations, some of them were big and dense black particles, while some others of them presented irregular hollow formations (Fig. 1.3a). In chloroplasts, Ca^{2+} concentration improved dramatically, and with the further expansion of granular thylakoid membranes, the distorted parts of membranes and the lamellar structures become vague while observed, and the increase of osmic acidophilic particle numbers took place in some chloroplasts (Fig. 1.3b). Some chloroplasts conglomerated with each other, forming a “butterfly configuration” (Fig. 1.3c). With relatively large amounts of Ca^{2+} particles mainly concentrated in the centre of nucleus, chromatins distributed in all directions around the nucleus (Fig. 1.3d). Observing Fig. 1.4a, it can be seen that cytosolic Ca^{2+} assembled in large quantities in certain cellular areas, and the occurrence of membrane breakages was found in some Ca^{2+} precipitate-centralized cellular areas. The serious chloroplast transformation was presented, and the black block masses with high electron density appeared after the partial breakage of some chloroplasts and the exosmosis of their stroma (Fig. 1.4b). With its collapsed and sectional vague membranes, the nucleus suffered from its serious transformation (Fig. 1.4c).

According to Fig. 2.1, it can be known that cellular after the ABA treatment to seedling leaves, cellular Ca^{2+} distribution changed significantly. The concentrations of Ca^{2+} precipitates decreased both in vacuoles

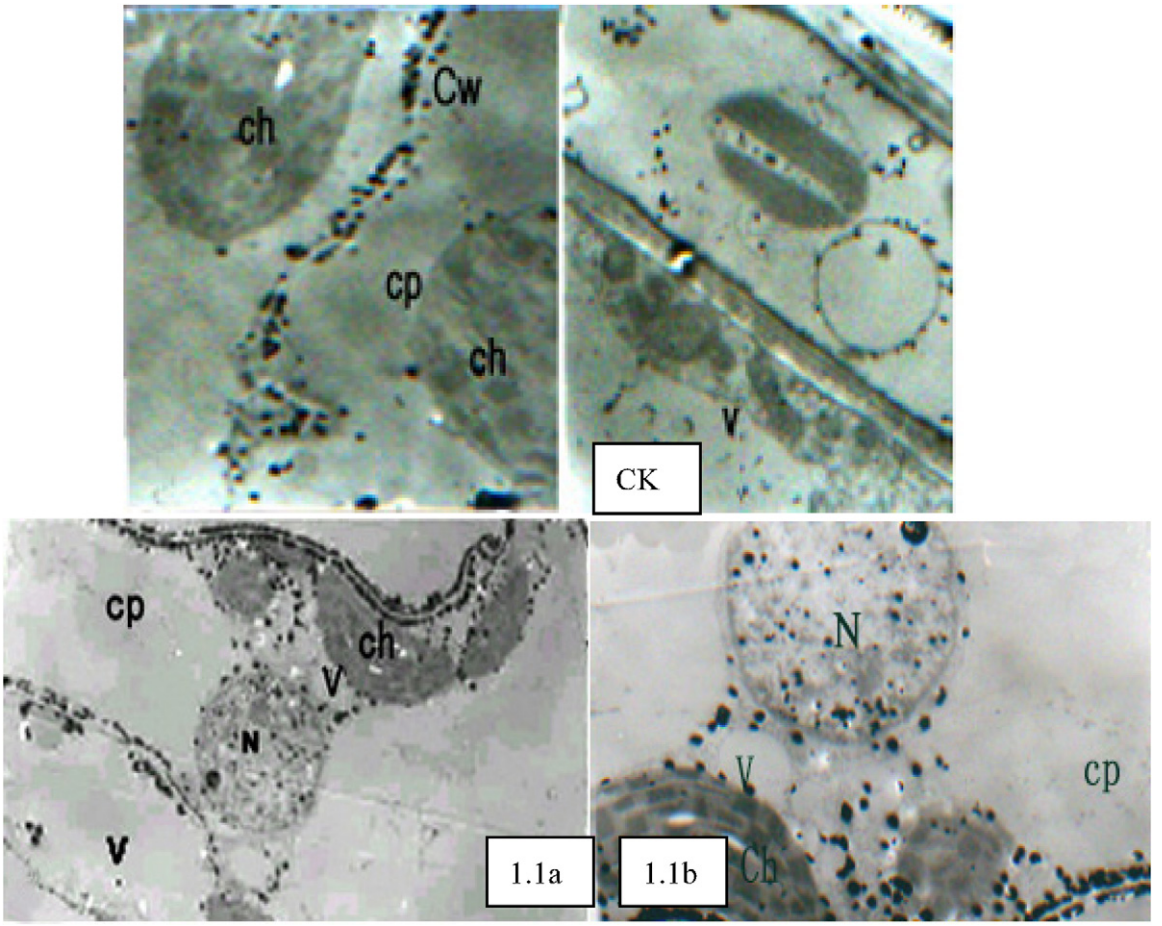
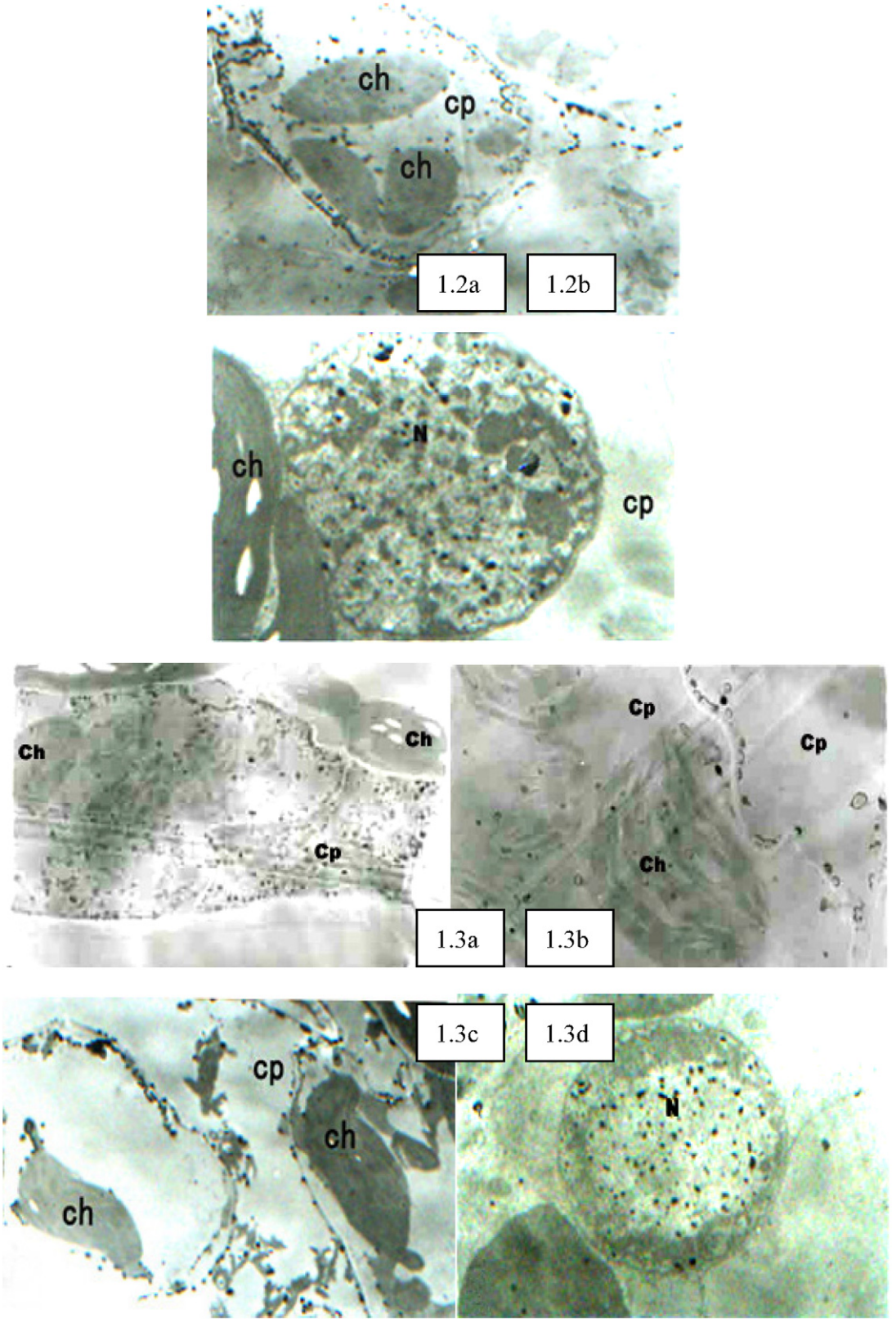


Fig. 1. The cellular Ca^{2+} distribution under PEG stress. CK (×5000), large amounts of calcium pyroantinonate precipitate particles can be observed in the intercellular gaps outside the cells. 1.1. PEG stress for 30 min (×6000): (a) Vacuolar Ca^{2+} concentration significantly decreased compared with CK. (b) Cellular super-microstructure remained unremarkable change compared with CK. 1.2. PEG stress for 45 min (×5000): (a) The cells were not significantly transformed. Relatively larger Ca^{2+} precipitates can be observed in the outsides of cell walls, and relatively huge amounts of Ca^{2+} precipitate particles can also be found in cytoplasm. Compared with 30 min PEG stress treatment (1.1), the precipitates under this treatment were disorderly and unevenly distributed, and considerably concentrated in certain parts of plant cells. (b) With the continuous elevation of Ca^{2+} concentration in the nucleuses and the occurrence of relatively large black particles, heterochromatin conglomerated. 1.3. PEG stress for 60 min (×6000): (a) With cell transformation, cytosolic Ca^{2+} concentration enhanced significantly, while the Ca^{2+} particles had irregularity in their configurations. (b) In chloroplasts, Ca^{2+} concentration improved dramatically. (c) Some chloroplasts conglomerated with each other, forming a "butterfly configuration". (d) With relatively large amounts of Ca^{2+} particles mainly concentrated in the centre of nucleus, chromatins distributed in all directions around the nucleus. 1.4. PEG stress for 75 min (×8000): (a) Cytosolic Ca^{2+} assembled in large quantities in certain cellular areas, and the occurrence of membrane breakages was found in some Ca^{2+} precipitate-centralized cellular areas. (b) Chloroplast transformed seriously, and the black block masses with high electron density appeared after the partial breakage of some chloroplasts and the exosmosis of their stroma. (c) Nucleus transformed seriously with its collapse membranes. Ch: chloroplast; Cp: cytoplasm; Cw: cell wall; N: nucleus; V: vacuole.

and outside cells, while huge amounts of Ca^{2+} precipitate particles can be observed in the internal sides of membranes with the dramatic enhancement of cytosolic Ca^{2+} concentration. Compared with ABA treatment, EGTA and ABA jointed treatment had quite different effects on the changes of cellular Ca^{2+} distribution in seedling leaves. From Fig. 2.3, it can be observed that cellular Ca^{2+} concentration increased slightly, while Ca^{2+} outside cells can not be seen because of the elimination of it caused by EGTA chelating (Fig. 2.2). After Verapamil (an inhibitor to Ca^{2+} channels) and ABA jointed treatment, the cellular Ca^{2+} accession from outside into inside was inhibited, because Verapamil interrupted the Ca^{2+} channels in the membranes, thus leading to the obviously-observed large quantities of Ca^{2+} distribution in both intercellular gaps and cell walls. While the cytosolic Ca^{2+} concentration did not increase significantly (Fig. 2.3). TFP (an inhibitor to CAM) af-



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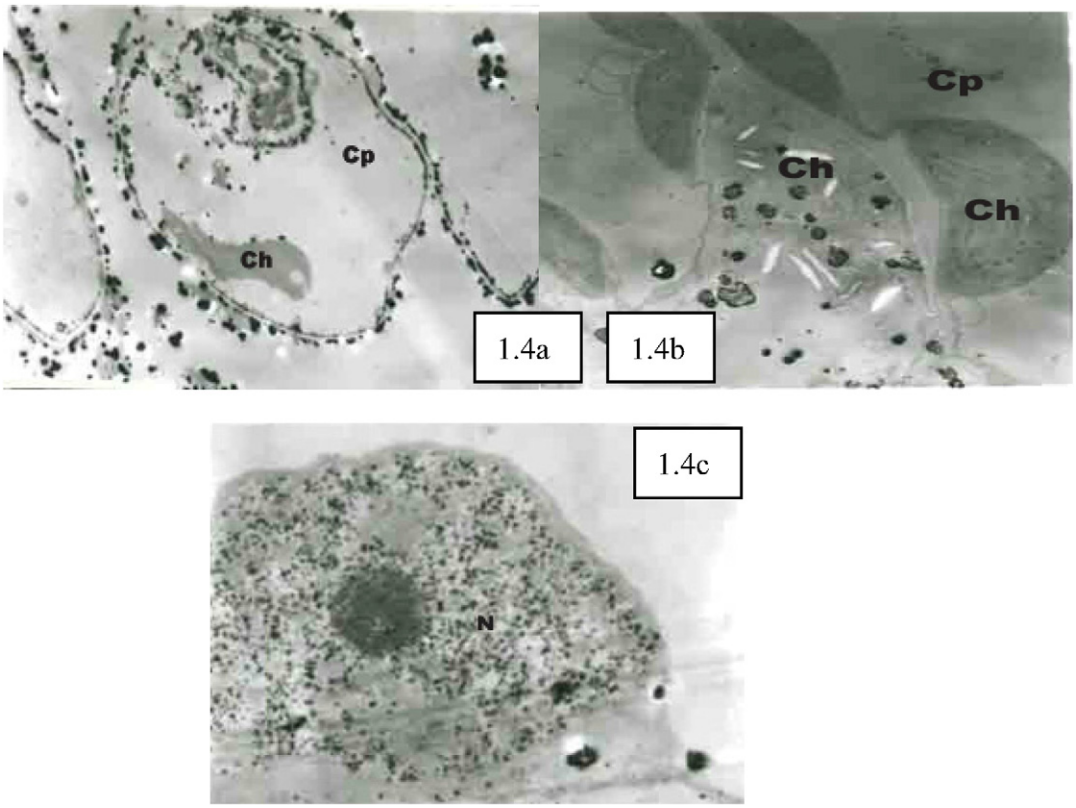

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fected the cellular Ca^{2+} accession from outside into inside to some extent. Many Ca^{2+} precipitates can be seen in intercellular gaps, while the trend of cytosolic Ca^{2+} concentration elevation was not significant than such trend observed under ABA treatment (Fig. 2.4). After EGTA treatment, the precipitates in the slice were eliminated. The original sectors for Ca^{2+} distribution such as intercellular gaps, 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. 2.5).

4. Discussion

The relationship between the nucleus of wheat seedling and Ca^{2+} under drought stress was examined with the cytochemical method of improved calcium antimonate precipitation. The results showed that there was a small concentration of Ca^{2+} in nucleus, the shape of the nucleus liked a ball and the chromatin was scattered distributed. The longer the duration of drought stress the higher the free Ca^{2+} concentration in nucleus and the more serious the ultrastructure of the nucleus, meanwhile, the membrane of the nucleus wrinkled and the chromatin seriously agglutinated, the shape of the nucleus was abnormal till its final disintegration [51–53]. It can be directly observed that under drought stress, the Ca^{2+} distributions and the cellular microstructures all changed in seedling leaves. At the early stage of drought stress, the nucleus and the chloroplast played important roles in regulating cellular Ca^{2+} concentrations, and the sectional distributions of Ca^{2+} in plant cells also provided vital roles in regulating the physiological activities regarding plant drought-resistance. Ca^{2+} participated in the processes of drought-stress and ABA-induced drought signal transduction as a pivotal medium. ABA could induce cytosolic Ca^{2+} concentration elevations in the mesophyll cells and guard cells of maize seedling leaves, such elevations originated mainly from extracellular Ca^{2+} sinks and partially from cellular Ca^{2+} sinks such as vacuoles. Extracellular CaM could participate in the process of drought signal transduction as an important medium through inducing the changes of cellular Ca^{2+} concentrations. It is also indicated and demonstrated in this experiment that under normal conditions, cellular free Ca^{2+} concentrations actually remain very low, and

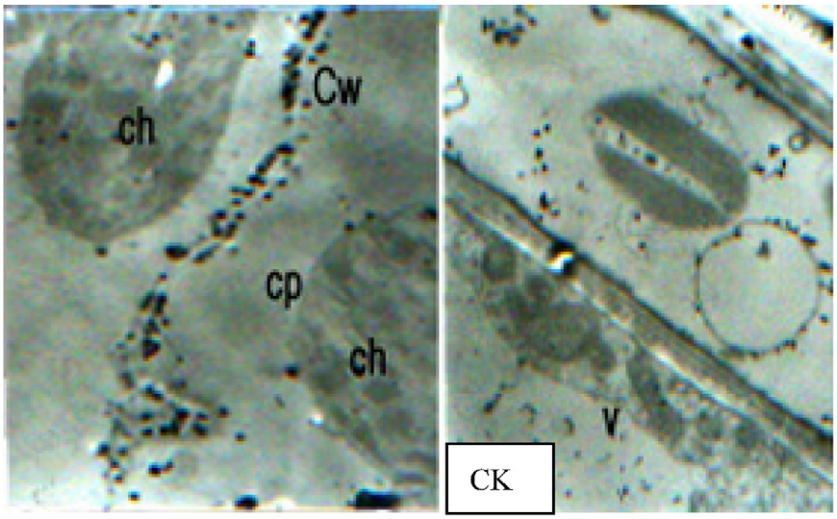

Fig. 2. The cellular Ca^{2+} distribution under ABA treatment, different jointed treatments and EGTA treatment. CK (×5000), large amounts of calcium pyroantinonate precipitate particles can be observed in the intercellular gaps outside the cells. 2.1. ABA treatment (×6000): Cellular Ca^{2+} distribution changed significantly compared with CK. 2.2. EGTA and ABA jointed treatment (×6000): Ca^{2+} outside cells can not be seen because of the elimination of it caused by EGTA chelating. 2.3. Verapamil and ABA jointed treatment (×6000): Large quantities of Ca^{2+} distribution in both intercellular gaps and cell walls can be obviously observed although cytosolic Ca^{2+} concentration did not increase significantly. 2.4. TPF and ABA jointed treatment (×8000): TFP affected the cellular Ca^{2+} accession from outside into inside to some extent, and many Ca^{2+} precipitates can be seen in intercellular gaps, while the trend of cytosolic Ca^{2+} concentration elevation was not significant than such trend observed under ABA treatment (2.1). 2.5. EGTA treatment (×10 000): The precipitates in the slice were eliminated. The original sectors for Ca^{2+} distribution such as intercellular gaps, chloroplasts, vacuoles, and plasma membranes presented transparent electron areas similar as the original precipitate structures. Ch: chloroplast; Cp: cytoplasm; Cw: cell wall; N: nucleus; V: vacuole.

vacuole serves as a main Ca^{2+} sink for higher plants. Identical to the results of chemical measurement, this experiment also proved that there were relatively large quantities of Ca^{2+} distributions in cell walls and among intercellular gaps.

It has been proved by a growing number of researches that the sectional distribution of Ca^{2+} in cells can be changed through altering the transportation of Ca^{2+} carriers in membranes and the status of cellular Ca^{2+} storage bodies [5–9,21–28,45–49,53–56], which can be stimulated by many exogenous and endogenous elements such as light, low and high temperatures, gravity, oxygen shortage, salt stress, hormone and gene-regulated physiological changes, thus further inducing a series of physiological and biochemical reactions in cells, and finally manifesting a change for the status of plant development and metabolism [22]. Therefore, the knowledge of the principles for plant signal transduction can be enriched and proved directly through determining the changes of cellular Ca^{2+} concentrations and understanding the spatial-temporal rules for such changes after cells' stimulus reception. Meanwhile, such researches can also provide some certain theoretical instructions to regulate plant development and metabolism.

In this experiment, it can be seen that membrane Ca^{2+} orderly distributed, and Ca^{2+} concentrations decreased dramatically in vacuoles and intercellular gaps 30 min after PEG stress. Therefore, it can be observed that the cytosolic Ca^{2+} partially originated from cells' outside and partially came from vacuoles (cellular Ca^{2+} sinks). With PEG stress continued, the cytosolic Ca^{2+} concentrations also elevated increasingly, and the cells transformed. 75 min after PEG stress, membrane breakage occurred in the sections where Ca^{2+} concentrated in large quantities. Numerous reports indicated that at the early stage of cold-stress, Ca^{2+} concentrations increased significantly in cytoplasm, but the concentrations of Ca^{2+} precipitates decreased in vacuoles and intercellular gaps. Therefore, it was speculated that under the stimulus of cold stress, Ca^{2+} in vacuoles and intercellular gaps can access into cytoplasm through Ca^{2+} transport channels in the membranes and other pathways as a second messenger, initiating and controlling relevant gene transcriptions and regulations aiming at cold stress resistance, thus leading to a series of physiological and biochemical reactions such as the occurrence of cold-tolerance relevant proteins, the enhancement of some enzymes' (including catalase, ATPase and so on) activities, and finally improving plant capacities of cold-resistance [50,65–73]. It can be speculated

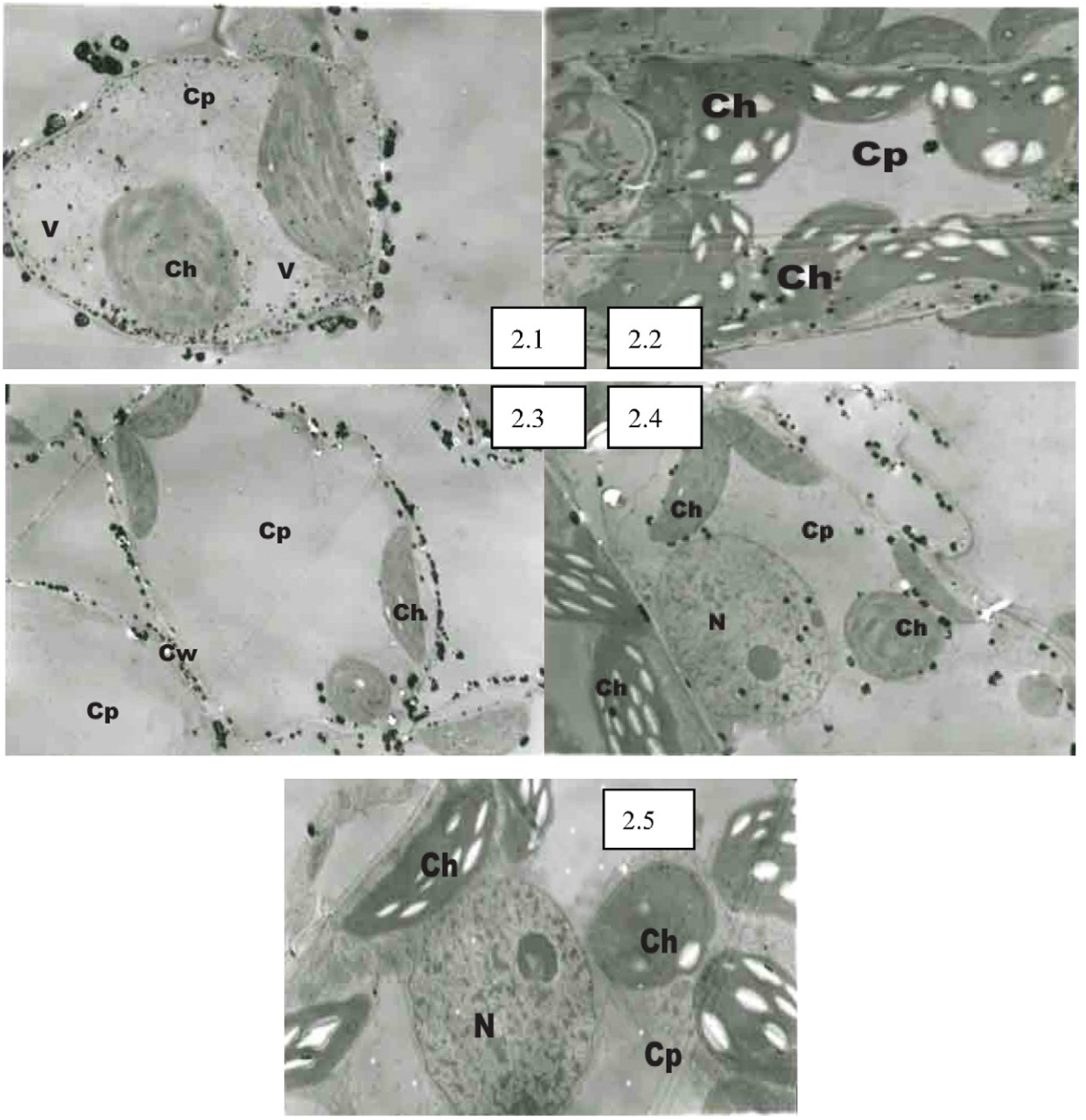

Fig. 2. (*continued*)

in this experiment that Ca^{2+} presents similar mechanism in plant drought and cold resistances, because the changes of Ca^{2+} distribution were identical both at the early stage of drought stress and under cold stress. It was also reported that Ca^{2+} applies such mechanism to enhance plant drought resistance as following: on one hand, Ca^{2+} can strengthen the stability of plant cellular structure through its direct effects; on the other hand, Ca^{2+} can also affect cellular protective systems, damage their functions and maintain the metabolic balance of such bio-radicals as active oxygen through its indirect effects [52]. Such report was also supported by the changes of Ca^{2+} distribution concluded in this experiment. The cytosolic Ca^{2+} concentrations and distributions can play active roles in regulating outside environments if they change in optimal extents. While if such changes overpass certain extents, they'll bring destructions and disturbs (such as the changes of cy-

toskeleton and membrane structure) to normal cellular structures and functions, altering membrane permeability and finally leading to the imbalance of cellular metabolism and the occurrence of disorders [39]. It was also found that the direct addition of Ca^{2+} carriers (such as A23187) or other elements to cell plant lines can lead to the elevation of cellular Ca^{2+} concentrations or the destruction of Ca^{2+} stable imbalance both inside and outside cells, thus further inducing cell death, demonstrating that there exist certain relationships between Ca^{2+} and cell death. In this experiment, with the advancement of drought stress, cytosolic Ca^{2+} concentrations continuously enhanced, and cells continuously transformed and finally disintegrated, which indicated that at the early stage of drought stress, the drought-resistance of cells enhanced with the elevation of cytosolic Ca^{2+} concentrations, while the increasing enhancement of Ca^{2+} concentrations may serve as an induction element for cell death during drought stress [67–73].

It was found in wheat seedling that under normal conditions, the chloroplast presented elliptical shapes with orderly-laminated granules and small amounts of Ca^{2+} distributions. With the advancement of drought stress, Ca^{2+} concentrations in chloroplasts increased, and chloroplasts transformed and finally disintegrated [52]. In another report, Zhong [51] also found the changes of chloroplast super-microstructures while observing the cellular super-microstructures in drought-stressed sugar-cane leave cells, which further clarified Wang's results. As important places for photosynthesis, chloroplasts can unavoidably produce such active oxygen as superoxide radicals during the process of photosynthesis. Under normal physiological conditions, the production and elimination of these radicals are balanced, bringing no damage to plants. While under drought stress, such balance can be destroyed, thus leading to the peroxidation of membrane lipids, causing membrane leakage and the destruction of cellular super-microstructures. In this experiment, the Ca^{2+} concentrations in chloroplasts increased because of chloroplasts' effects on cytosolic Ca^{2+} concentration regulation as Ca^{2+} pumps, and the increased Ca^{2+} concentrations in chloroplasts are also beneficial for the stabilization of their membranes. However, with the advancement of drought stress, chloroplasts' structures were destroyed, especially that the transformation of their granular laminas may make great contributions to cell death. The membrane structures in chloroplast granular laminas play pivotal roles in luminous energy absorption, transmission and conversion. So, the damage of such structures may lead to metabolic disorders and finally result in cell death [27–35,57–62].

At the early stage of drought stress, despite of the elevation of Ca^{2+} concentrations in nucleus, the structures of nucleus remained stable, which demonstrated the roles nucleus played in cytosolic Ca^{2+} concentration regulation. However, with the continuous elevation of Ca^{2+} concentration, chromatins conglomerated and nucleus membranes disintegrated. Through conducting the research of the effects of Ca^{2+} on the reconstruction of non-cellular nucleus systems, Zhao and Zhang [52] found that the addition of EGTA to Ca^{2+}-contented samples led to the elimination of chromatin conglomeration in striping sperms, while without such EGTA addition, the chromatins were highly-conglomerated in Ca^{2+}-contented striping sperms. Therefore, it can be speculated that high Ca^{2+} concentrations may play inhibited roles in the elimination of chromatin conglomeration. Such speculation can be expressly proved through observing the TEM slices produced in this experiment. Zhao's experiment results were also proved by Wang's reports (Wang and Zhang [50]) regarding the researches of the relationships between Ca^{2+} and the changes of nucleus super-microstructures in wheat seedling leaves under drought stress, which is similar to the results observed in this experiment.

It was showed that although various stresses can all lead to the increase of cytosolic Ca^{2+} concentrations, they have different origins for Ca^{2+} concentration increase. In the heat-shock reactions of tobacco, the increase of cytosolic Ca^{2+} concentrations originated from two Ca^{2+} sinks inside and outside the cells, respectively. While in the process of low temperature-induced alfalfa cold acclimatization, such increase originated from Ca^{2+} sinks outside the cells, and Ca^{2+} can be released from cellular Ca^{2+} sinks even through touch and wind. In this experiment, after the implementation of EGTA, Verapamil and TFP pretreatments, although ABA could induce the elevation of cytosolic Ca^{2+} concentrations, the extents for such elevation were significantly lower than such extents under only-ABA induction without pretreatments. Therefore, it can be directly concluded that ABA-induced elevation of cytosolic Ca^{2+} concentrations originated mainly from Ca^{2+} sinks outside sells and partially from cellular Ca^{2+} sinks. Ca^{2+} signals can be created directly from Ca^{2+} sinks outside cells and released from cellular Ca^{2+} sinks, or it can also be jointly produced by both types of Ca^{2+} sinks. Currently, pharmacological experiments are mainly conducted for the investigation of the origin of Ca^{2+} concentration elevation due to technological limitations. It has been clarified that the

changes of cytosolic Ca^{2+} concentrations mainly resulted from Ca^{2+} released from cellular Ca^{2+} sinks in such aspects of experiments as pollen tube growth [24], oxygen shortage [19], cold-shock [30] and so on. While as to the researches regarding plant defensive reactions, almost all the results manifested that Ca^{2+} outside cells played paramount roles as showed in the experiments applying such plant materials as soybean, carrot, *Petroselinum sativum* and tobacco. In these experiments, Ca^{2+} concentrations lowered, leading to the partial inhibition of phytoalexin synthesis [56]. However, the elevation of Ca^{2+} concentrations induced by cold stimulation, drought stress, oxygen shortage and salt stress mainly originated from the joint effects of extracellular Ca^{2+} influx and release of Ca^{2+} from cellular Ca^{2+} sinks [41–46]. Through TEM, it can also be observed in this experiment that the elevation of cytosolic Ca^{2+} concentrations could be induced by 50 μM ABA treatments to seedling leaf cells, while if extracellular Ca^{2+} influx was blocked by other reagents, the elevation of cytosolic Ca^{2+} concentrations could also be inhibited to some extents, showing that ABA-induced cytosolic Ca^{2+} concentrations elevation mainly originated from extracellular Ca^{2+}. Contrary to the results of TEM observation, under digital con-focus microscopic observation, ABA-induced Ca^{2+} concentrations were not affected by any element 30 min after 100 μM Verapamil pretreatments. The following two possibilities could lead to such results: (1) different Ca^{2+} channels in the membranes have different sensitivities to Verapamil; or (2) ABA-induced Ca^{2+} concentrations elevation could originated from the release of Ca^{2+} from cellular Ca^{2+} sinks, which can be demonstrated by a 2 mM EGTA pretreatment experiment in maize cuticles. The results of such experiment showed that ABA-induced Ca^{2+} concentrations elevation could be inhibited by EGTA, indicating that such elevation might mainly originate from extracellular Ca^{2+} influx [1–5,31–36].

As a small protein molecule with a molecular weight of 17 kDa, CaM is universally-existed in plants. In this experiment, ABA-induced cytosolic Ca^{2+} concentrations elevation was inhibited by TFP, which represents an inhibitor to CaM and its analogues, showing that CaM participated in ABA signal transduction. Then, how could TFP inhibit ABA-induced cytosolic Ca^{2+} concentrations elevation through antagonizing CaM? As widely existing in plant cells as well as cell walls, cellular CaM serves as the most important Ca^{2+} receptor protein for initiating downstream Ca^{2+} signal events. In this experiment, through applying TFP, only the downstream Ca^{2+} signal events could be affected, while the changes of Ca^{2+} concentrations could not be blocked. Therefore, the effects of TFP on the pathway of ABA signal transduction could only be implemented through antagonizing CaM in cell walls. It was also proved in the experiment applying broad bean cuticle cells that Ca^{2+} concentrations could be elevated by extracellular CaM, and therefore CaM was considered to have the functions of signal molecules as extracellular signal elements, which could cause the changes of Ca^{2+} concentrations [66–73]. In this experiment, it was also showed that ABA could induce and transmit the signals of Ca^{2+} concentrations elevation through extracellular CaM.The total report here clearly provided a direct evidence for calcium ion as an important signal. However, the related molecular mechanism for ABA's correlation with extracellular CaM in inducing the changes of Ca^{2+} concentrations remains to be further studied and clarified at the next step.

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