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Spatial and Temporal Calcium Signaling and Its Physiological Effects in Moso Bamboo under Drought Stress

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Abstract: Elevations in cytosolic free calcium concentration constitute a fundamental signal transduction mechanism in plants; however, the particular characteristics of calcium ion (Ca^{2+}) signal occurrence in plants is still under debate. Little is known about how stimulus-specific Ca^{2+} signal fluctuations are generated. Therefore, we investigated the identity of the Ca^{2+} signal generation pathways, influencing factors, and the effects of the signaling network under drought stress on *Phyllostachys edulis* (Carrière) J. Houz. Non-invasive micro testing and laser confocal microscopy technology were used as platforms to detect and record Ca^{2+} signaling in live root tip and leaf cells of *P. edulis* under drought stress. We found that Ca^{2+} signal intensity (absorption capacity) positively correlated with degree of drought stress in the *P. edulis* shoots, and that Ca^{2+} signals in different parts of the root tip of *P. edulis* were different when emitted in response to drought stress. This difference was reflected in the Ca^{2+} flux and in regional distribution of Ca^{2+}. Extracellular Ca^{2+} transport requires the involvement of the plasma membrane Ca^{2+} channels, while abscisic acid (ABA) can activate the plasma membrane Ca^{2+} channels. Additionally, Ca^{2+} acted as the upstream signal of H_2O_2 in the signaling network of *P. edulis* under drought stress. Ca^{2+} was also involved in the signal transduction process of ABA, and ABA can promote the production of Ca^{2+} signals in *P. edulis* leaves. Our findings revealed the physiological role of Ca^{2+} in drought resistance of *P. edulis*. This study establishes a theoretical foundation for research on the response to Ca^{2+} signaling in *P. edulis*.

Keywords: Ca^{2+} signal; drought stress; living cell; Moso Bamboo (*Phyllostachys edulis*); plasma membrane Ca^{2+} channels; signal network

1. Introduction

Calcium ions (Ca^{2+}) are a primary signaling element for diverse cell processes in response to environmental cues. Ca^{2+} is a vital regulatory molecule for response to stress in plant growth and development [1,2]. When plants are affected by various physical stimuli, such as temperature, drought, salt, light, gravity, or chemical substances, such as plant hormones and pathogenic inducers, extracellular and intracellular sources of calcium release Ca^{2+} into the cytoplasm through Ca^{2+} channels. The spatio-temporal activity of membrane-localized Ca^{2+} channels or transporters causes an increase in cytosolic free calcium ion concentration (Ca^{2+})cyt, resulting in specific signals [3,4]. Almost all of the extracellular stimuli can lead to changes in intracellular (Ca^{2+})cyt. However, different stress stimuli can lead to variations in the pattern of Ca^{2+} spatial-temporal changes. There are significant differences in time, frequency, amplitude, and regional distribution. Studies have illustrated that plants may rely on different forms of Ca^{2+} to reflect the specificity of different stimulation signals to achieve signal transduction [5].

Although Ca^{2+} signaling has been extensively studied in other gramineous plants, such as rice, little is known about the Ca^{2+} signal identities and functions of the clonal plant *Phyllostachys edulis* (Carrière) J. Houz. Therefore, a better understanding of the calcium signal characteristics of bamboo under drought stress is an important prerequisite for the study of clonal habits of bamboo using signal transduction methods.

At present, research has shown that root tip cells of *P. edulis* seedlings transport Ca^{2+} from the extracellular region, cell walls, nucleus, and other calcium stores under drought stress. With increased duration of drought stress, the distribution of and changes in Ca^{2+} will produce regular fluctuations [6]. The more pronounced the drought stress, the greater the Ca^{2+} distribution in the root tip. Exogenous application of calcium fertilizer can relieve the physiological effects of drought stress and improve drought resistance in *P. edulis* [7]. Although roots constitute the most direct organ of water absorption, both Ca^{2+} uptake velocity and density of the different organ changed under drought stress [8]. Moreover, stomatal opening and closing behaviors were also regulated by Ca^{2+} signals at the cellular scale [9].

When there are no environmental pressures present, the majority of the Ca^{2+} in plant cells is distributed in the extracellular compartments, cell wall, vacuoles, and endoplasmic reticulum, with less concentrated distribution in the cytoplasm to prevent the precipitation of calcium and phosphoric acid [10]. To regulate this lower Ca^{2+} concentration in the cytoplasm, plant cells will actively export Ca^{2+}. When a stimulus signal reaches a cell, plasma Ca^{2+} channels transiently increase Ca^{2+} permeability. When the cytoplasmic Ca^{2+} concentration increases to a certain threshold, it binds to calmodulin (CaM) to form Ca-CaM compounds, and thus activate CaM. Activated CaM further activates various key enzymes in the plant, which further phosphorylates and dephosphorylates phospholipase, nicotinamide adenine dinucleotide (NAD) kinase, and Ca^{2+}-ATPase. In addition, it amplifies the initial stimulation signal and subsequently causes the cells to produce a physiological response corresponding to the signal, such as cell division, material synthesis, etc. [2,11].

Under drought stress, a complex signaling network is formed by a communication mechanism between regulatory signals. Ca^{2+} signaling can be combined with calcium receptors, such as CaM, to amplify the signal and transmit the oscillation to initiate stomatal closure and production of reactive oxygen species [12,13]. Previously, reactive oxygen species were considered toxic byproducts of plant metabolism. However, recent studies have shown that reactive oxygen species also have an important part to play in cellular signal transduction and regulation networks [14–16]. Abscisic acid (ABA) is a root chemical signal that plays an important role in regulating stomatal movement of plants under drought stress. H_2O_2 can be used as a downstream signal of ABA to activate Ca^{2+} channels in the plasma membrane. Involvement of ABA can induce stomatal closure by increasing Ca^{2+} concentration in guard cells [17]. Sha et al. used 25% PEG-6000 to simulate water stress in maize plants, inducing CaM gene expression in the leaves [18]. The study found that exogenous ABA treatment can also induce significant CaM gene expression, and that H_2O_2 is involved in ABA-induced CaM gene expression in the late regulation period.

The emergence of new technologies has made it possible to study changes in Ca^{2+} signaling in response to environmental stress in plants. Non-invasive microelectrode technology (NMT) and laser confocal microscopy are effective techniques for detecting Ca^{2+} signals. Antoine et al. demonstrated the influx of Ca^{2+} over the course of fertilization in maize with non-invasive microelectrode technology [19]. In addition, a live-cell Ca^{2+} imaging platform has been used to detect Ca^{2+} signals in the cytoplasm and nucleus of *Arabidopsis thaliana* (L.) Heynh. This technique was also used to observe the spatial-temporal distribution of Ca^{2+} in living cells of *A. thaliana* under stimulated adverse environmental conditions [20]. These studies use a method to generate Ca^{2+} signaling pathways by treating experimental materials with Ca^{2+} inhibitors. Ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), lanthanum chloride ($LaCl_3$), and chlorpromazine (CPZ) are the most commonly used reagents. At present, laser confocal scanning microscopy has been used to study Ca^{2+} fluorescence localization in *P. edulis* cells under drought stress; however, most of these studies have been limited to the root tip [7]

and few studies have been conducted on Ca^{2+} localization in cells located in the leaves of *P. edulis* using this technique.

To bridge this scientific gap, in this study we investigated the spatial-temporal location and flux velocity of Ca^{2+} ions by inducing drought stress in *P. edulis* with 20% PEG-6000. Using non-invasive micro-test technology (NMT) and laser confocal microscopy, we demonstrated the regularity of cellular Ca^{2+} dynamics in response to drought stress and provided measures of Ca^{2+} signaling in *P. edulis* leaf cells. We further studied the Ca^{2+} signaling pathway and analyzed the communication of the signal network pathway between Ca^{2+}, H_2O_2, and ABA in leaf cells using Ca^{2+} inhibitors (Ca^{2+} channel blockers) and ABA. The aim of this study was to reveal the physiological role of Ca^{2+} in drought resistance and establish a theoretical foundation for the cellular response to Ca^{2+} signaling in *P. edulis*.

2. Materials and Methods

2.1. Plant Materials and Treatment

The sprouting seed materials used in this study were taken from the parent *P. edulis* from Guilin, Guangxi in September 2017. The thousand seed weight of these seed materials is equal to 22.75 ± 0.35 g. The *P. edulis* seeds were treated in November 2017. The seeds were soaked in warm water for 24 h at 50 °C, and then removed and disinfected with 5‰ potassium permanganate solution for 5 min. The sterilized seeds were then repeatedly washed with distilled water and germinated in an incubator in a dark environment set to a constant temperature of 25 °C. After the seeds germinated, the seedlings were selected for regular, even growth and placed in a Petri dish with pad disinfectant (lower layer) and filter paper (upper layer). The dishes were cultured at a constant temperature of 28 °C in an illumination incubator (PRX-1000B, Safe, Ningbo, China). The Petri dishes were set to point the roots vertically downwards. Proper humidity was maintained in the Petri dishes and any seeds found to be growing mold were removed. When the vertical root length reached approximately 3 cm, the seedlings were transferred to a Seed Germination Pouch (Phytotc CYG-98LB, size: 30 cm × 25 cm, Beijing Bioconsumable Tech., Ltd., Beijing, China) and cultured with a 12-h light period with a light intensity of 120–150 mmol m^{-2} s^{-1} and a temperature of 22 ± 2 °C. Figure 1 gives more details about the performance of the experimental materials.

Figure 1. Cultivation of *P. edulis sprouting* seedlings.

For each experimental treatment, ten strains of *P. edulis* seedlings were selected in duplicate. To substitute drought stress, 20% polyethylene glycol-6000 (PEG, Coolaber, Beijing, China) was used for 5 min, 10 min, 15 min, and 30 min. Distilled water was used for the control (CK). In addition, Ca^{2+} inhibitors and ABA (Sigma-Aldrich, Shanghai, China) were used to treat *P. edulis* seedlings. The Ca^{2+} production pathway and its distribution were studied, and the effects of Ca^{2+} signaling on the H_2O_2/ABA signaling network were analyzed under drought stress. The Ca^{2+} inhibitors used in

the experiment were extracellular Ca^{2+} chelating agent EGTA, Ca^{2+} channel blocker $LaCl_3$, and CaM antagonist CPZ (Sigma-Aldrich, Shanghai, China). The dosage of additives was added according to the method of Lu [21].

2.2. Laser Confocal Microscopy Luminescence Imaging

Here, we present detailed instructions for laser confocal microscopy luminescence imaging of cytosolic Ca^{2+} and H_2O_2 concentration and distribution in root tip and leaf cells of *P. edulis* seedlings.

2.2.1. Esterified Fluorescent Probe Stock Solution Configuration

The calcium ion fluorescent probe Fluo-8 (AAT Bioquest, Sunnyvale, CA, USA) was fully dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Shanghai, China) to a concentration of 1 mmol/L, −20 °C dark storage reserve.

2′,7′-dichloro fluorescin diacetate (H2DCFDA, Sigma-Aldrich, Shanghai, China) was made up of 50 mmol/L DMSO mother liquor, which was stored in a separate container and frozen.

2.2.2. Fluorescent Labeling

As to the root tip cells and leaf cells Ca^{2+} fluorescent labeling, we put the lower epidermis of the leaves of *P. edulis* seedlings flat on transparent tape and used a surgical blade to gently scrape the upper epidermis off the leaves. Then we placed the samples of lower epidermis in Hank's balanced salt solution (without calcium ions) containing 20 μmol/L Fluo-8. We incubated them for 40 min in the dark at room temperature and rinsed the lower epidermis of *P. edulis* leaves with a buffer several times. We then incubated them again at room temperature for 20 min, ensuring that the esterification probe was fully dissolved. Finally we placed them on a glass slide and added 0.5 mL of Hank's buffer to complete slice preparation.

As to the H_2O_2 fluorescent labeling of *P. edulis* leaf cells, the lower epidermis of *P. edulis* seedling leaves was first laid flat on transparent tape, and then the upper epidermis of the leaves was removed using a surgical blade and placed in MES, Free acid, monohydrate (Coolaber, Beijing, China) buffer to a final concentration of 50 μmol/L and incubated at room temperature in the dark for 15 min. We rinsed the lower epidermis samples several times with a buffer and placed them on glass slides. We then added 0.5 mL of buffer to complete slice preparation.

2.2.3. Laser Confocal Microscope Observation

Localization of Ca^{2+} fluorescence in *P. edulis* root tips and leaves: The prepared in vivo test slices were placed on a laser confocal microscope (LSM510, LeicaDM4, Berlin, Germany) for observation and scanning. Fluorescence intensity was controlled and all parameters were kept constant during the test (parameter settings: excitation wavelength 488 nm, BP 505-530, Pinhole 280, DG 581, AO 0.1, AG 1.34). At least three fields from different repeats were selected for each test. After the results were stable, one field of view was used for the analysis.

Localization of H_2O_2 fluorescence in root tips and leaves of *P. edulis*: The prepared in vivo test slices were placed on a laser confocal microscope (LSM510) for observation and scanning (parameter settings: excitation wavelength 488 nm, BP 505-530, Pinhole 386, DG 768, AO 0, AG 1). At least three fields from different repeats were selected for each test. After the results were stable, one field of view was used for analysis.

2.3. Measurement of Ca^{2+} Flux

Net Ca^{2+} flux was measured using non-invasive micro-test technology (Physiolyzer, Younger USA LLC, Amherst, MA 01002, USA; Xuyue (Beijing) Sci. & Tech. Co., Ltd., Beijing, China). NMT non-invasively measures Ca^{2+} fluxes with a high temporal and spatial resolution. It measures

the concentration gradient of Ca^{2+} by means of selective microsensor oscillation between two points in the root tip of *P. edulis* seedlings (Figure 2).

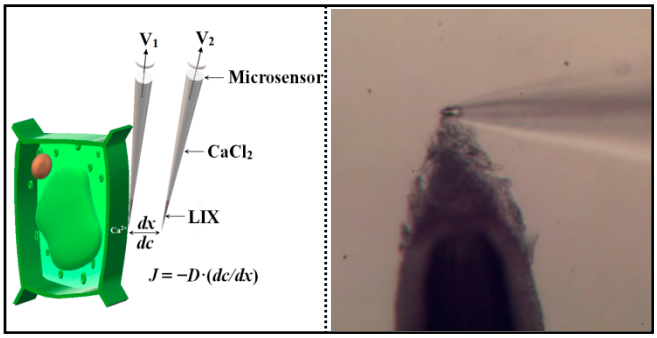

Figure 2. Schematic diagram and measured photo of Ca^{2+} flux analysis by non-invasive micro-tests. The diagram is to demonstrate the principles behind NMT testing and is not to scale. The tip diameter of the microsensor is about 5 μm.

After different test treatments, the roots were rinsed with redistilled water and immediately incubated in measuring solution to equilibrate for 10 min. Then, the roots were transferred to a measuring chamber containing 10−15 mL of a fresh measuring solution. Ions were monitored in the following solutions: 0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM NaCl, 0.3 mM MES, and 0.2 mM Na_2SO_4, following adjustment of the pH to 6.0. The measuring chamber was mounted on the micromanipulator, and the flux microsensor was positioned close to the root tip at four points: at the pileorhiza, meristematic zone, elongation zone, and mature zone (0 μm, 200 μm, 500 μm, and 800 μm from the root apex) of *P. edulis* seedlings.

The system setup parameters in the experiment are as follows. The Ca^{2+} flux microsensor (Φ4.5 ± 0.5 μm, XY-CGQ-01, Xuyue (Beijing) Sci. &Tech. Co., Ltd., Beijing, China) was filled with a backfilling solution (100 mM $CaCl_2$) to a length of approximately 1.0 cm from the tip. The micropipettes were front filled with 40–50 μm columns of selective liquid ion-exchange cocktails (Ca^{2+} LIX, XY-SJ-Ca, YoungerUSA LLC, Amherst, MA, USA). An Ag/AgCl wire microsensor holder YG003-Y11 (Younger USA) was inserted in the back of the microsensor to make electrical contact with the electrolyte solution. YG003-Y11 (Younger USA) was used as the reference microsensor. Prior to the flux measurement, the flux microsensor was calibrated with a measuring solution having different concentrations of Ca^{2+} 0.1 mM and 0.01 mM. The electrodes with a Nernstian slope > 22 mV per decade were used in this study. Only the same flux microsensor was calibrated again according to the same procedure and standards after each test. Data was discarded if the post-test calibrations failed. The following figure shows the schematic and actual measurement of Ca^{2+} flux.

The data for Ca^{2+} fluxes were calculated by Fick's law of diffusion as follows:

$$J = -D \cdot (dc/dx) \quad (1)$$

where dx (30 μm) is the distance the flux microsensor moved repeatedly from one point to another perpendicular to the surfaces of the samples at a frequency of ca. 0.3 Hz.

2.4. Statistical Analysis

Data were analyzed by single factorial analysis of variance and statistical correlation analysis. The significance of differences among means was evaluated using the least significant difference test, with a family wise error rate of 0.05, using the Statistical Package for Social Sciences, v18.0 (SPSS Inc., Chicago, IL, USA). Significant differences are marked with alphabet.

3. Results and Discussion

3.1. Variation in the Flux and Distribution of Ca^{2+} among Different Parts of the Root Tips of P. edulis Seedlings under Drought Stress

The transient net Ca^{2+} flux was measured from different regions along the root axis using NMT in four different areas, the root apex and root hair zone, including pileorhiza, the meristematic zone, elongation zone, and mature zone (Figure 3a). Responses in the root apex Ca^{2+} flux are shown in Figure 3b. The results showed that there was a significant difference in the ability of *P. edulis* seedlings to absorb Ca^{2+} from different parts of the root tip after drought stress. The Figure 3b showed the most uptake of Ca^{2+} in pileorhiza. The pileorhiza was the area with the strongest Ca^{2+} signal response ability, and the Ca^{2+} concentration in the root pileorhiza was the highest. The elongation zone was the region with the strongest Ca^{2+} efflux. The intensity of Ca^{2+} uptake capacity at different parts of the root tip from strong to weak was: pileorhiza, mature area, meristematic zone, and elongation zone. The Figure 3c shows the fluorescence localization of *P. edulis* root tip cells by laser confocal microscopy. To better observe the distribution of Ca^{2+} in the pileorhiza, the corresponding heat map is shown in Figure 3d. Combined with Figure 3b, it can be concluded that there were differences in the responsiveness of different parts of the root tip of *P. edulis* to drought stress, which is reflected in both the Ca^{2+} flux and regional distribution.

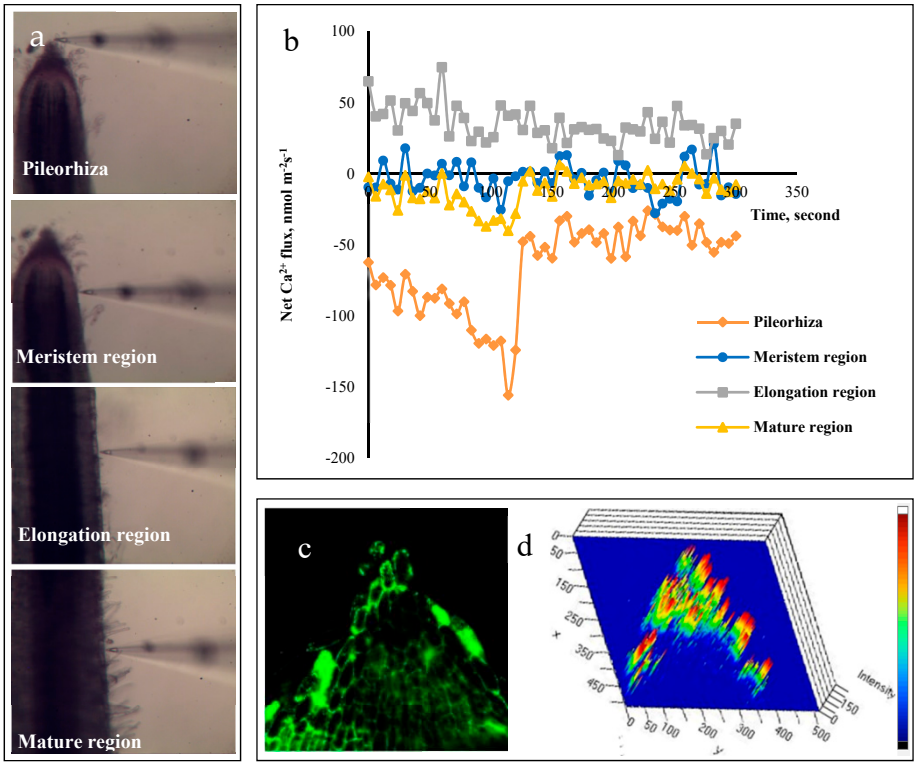

Figure 3. The flux and distribution of Ca^{2+} in different parts of *Phyllostachys edulis* root tips under drought stress. (**a**,**b**) Net Ca^{2+} fluxes in different parts of root tip of *P. edulis* seedlings under drought stress induced by 20% PEG for 10 min. At each position, an average Ca^{2+} flux was measured for 5 min before the electrode was repositioned (Data Repetition: 6 replicates). (**c**) Green fluorescence intensity is positively correlated with the Ca^{2+} concentration. The Ca^{2+} fluorescence localization at the root tip of the *P. edulis* seedlings treated with PEG-simulated drought stress for 10 min. (**d**) A heat map of a further analysis for (**c**), which clearly presented that the concentrated distribution of Ca^{2+} was in the pileorhiza of the *P. edulis* root tip cells under drought stress.

We confirmed this difference in the results of Ying [7], which used laser confocal microscopy on fluorescence localization in the root tip. This study showed that Ca^{2+} absorption intensity in the elongation zone was weakest under drought stress, while Ying's study showed that under drought stress, the Ca^{2+} in the root tip of *P. edulis* was mainly distributed in the pileorhiza and elongation zone; the distribution in the meristem area was relatively lower. This was due to the presence of a large number of small vacuoles in the cells of the elongation zone, which were not present in meristem zone. The vacuoles, as a calcium bank in plant cells, may provide a large amount of Ca^{2+} for *P. edulis* cytoplasm under drought stress. This leads to a greater concentration of Ca^{2+} in the elongate zone.

3.2. Effects of Drought Stress Duration on Ca^{2+} Absorption Regularity in the Pileorhiza of P. edulis

Under different durations of PEG-induced drought stress, we observed different conductivity, which represents leaf cell permeability (Figure 4a). An increase in drought stress duration caused electrolyte leakage in the cells, which in turn led to an increase in leaf conductivity. The cell membrane permeability of *P. edulis* seedlings increased, and there was a positive correlation between time and membrane permeability. The difference in conductivity increase between the 10- and 15-min treatments was higher than that in the 5–10 min period (difference in conductivity = 15.2%). The relative electrical conductivity of *P. edulis* seedlings under drought stress was 40.4% higher in the 30-min treatment than in the 15 min treatment. We observed significant correlation between Ca^{2+} flux and drought stress time ($p < 0.01$, Pearson correlation coefficient (r) = 0.967) (Figure 4b). All the flux data in Figure 4b represents stable and optimal real-time flux that can respond to Ca^{2+} concentration in response to drought stress. Relative to the control group, Ca^{2+} flux in pileorhiza of *P. edulis* seedlings gradually shifted from efflux to influx. Ten minutes of PEG treatment was found to be a "threshold", after which efflux became influx. There was also a positive correlation between leachate conductivity and time as Ca^{2+} absorption intensity in the pileorhiza ($p < 0.01$, $r = 0.976$), and leaf membrane permeability increased.

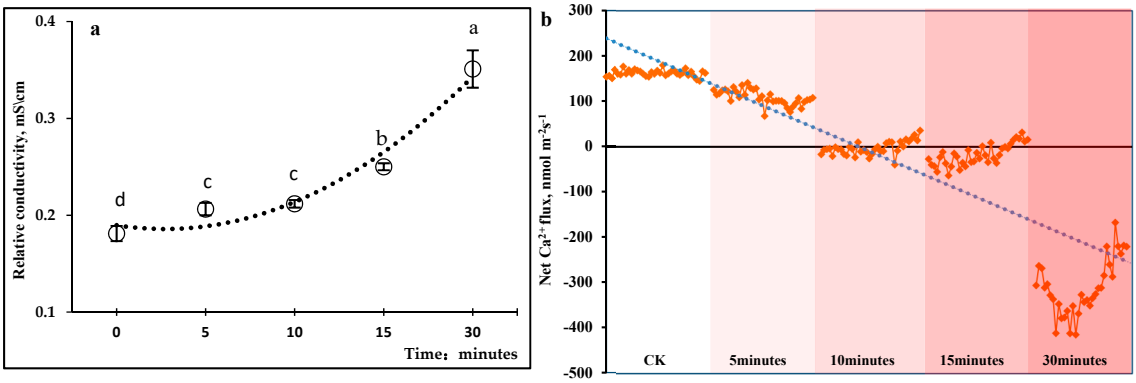

Figure 4. Ca^{2+} flux of the pileorhiza cells of *P. edulis* seedlings under different durations of PEG-treatment. (**a**) The relative conductivity of leaves from *P. edulis* seedlings treated with PEG-simulated drought stress for 5 min, 10 min, 15 min, and 30 min (Data Repetition: 6 replicates). The control (CK) is represented by 0, which was a control group incubated with distilled water. The dashed curve ($y = 0.0132x^2 - 0.0406x + 0.2171$, $R^2 = 0.9615$) represents the linear regression model for relative conductivity and PEG-simulated drought stress. (**b**) The depth of the red color represents the length of treatment time with 20% PEG simulating drought stress. From left to right in Figure 4b: the CK group, PEG-simulated drought stress treatment for 5 min, 10 min, 15 min, and 30 min, respectively. The flux data for each treatment in the figures reflected the steady-state real-time flux of maximum response to Ca^{2+} under drought stress (Data Repetition: 3 replicates); each segment of real-time data comprises flux data for three minutes.

Ca^{2+} flux in the pileorhiza of *P. edulis* seedlings in the control group (CK) maintained an out-of-range value of approximately 152 nmol m^{-2} s^{-1}. Figure 5 showed that Ca^{2+} flux in the pileorhiza was only slightly changed in the experimental group after 5 min of PEG treatment, indicating that the

slight degree of drought stress did not significantly induce the stress response leading to Ca^{2+} signaling in such a short time. When treated with PEG for 30 min, the Figure 5 shows a "V-shaped" pattern of ion uptake, with net flux first increasing and then decreasing. As drought stress time increased, Ca^{2+} flux gradually returned to control group standard of efflux. It is evident that there is a positive correlation between Ca^{2+} signal and drought stress time (Figure 5). Under drought stress, there is a "stress threshold" for the Ca^{2+} signal response. When the stress level reached this threshold, the Ca^{2+} flux at the pileorhiza underwent significant changes, which may be related to the plant's ability to withstand drought stress.

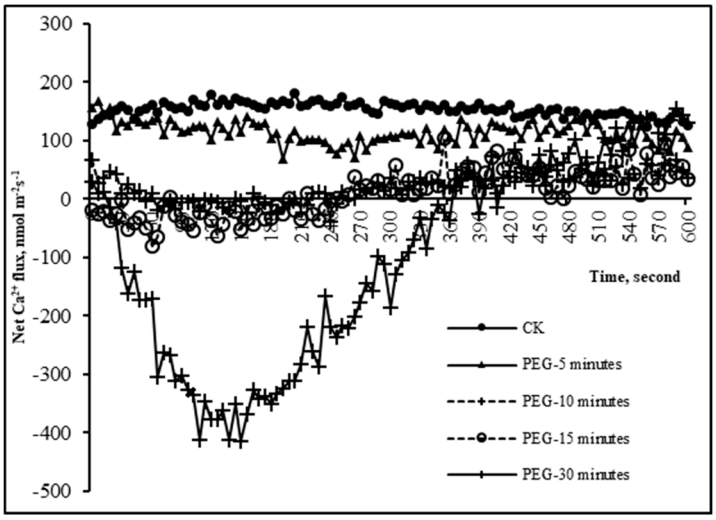

Figure 5. Changes in net Ca^{2+} flux in the pileorhiza of *P. edulis* seedlings after PEG-induced drought stress. Real-time Ca^{2+} flux by the pileorhiza of by PEG-induced drought stress for 5 min, 10 min, 15 min, 30 min, and CK, respectively (Data Repetition: 3 replicates).

Thirty minutes of PEG simulated drought stress showed obvious Ca^{2+} absorption in the pileorhiza, and Ca^{2+} flux oscillation increased. With the increase in the duration of drought stress, Ca^{2+} absorption increased. *P. edulis* seedlings were able to absorb Ca^{2+} from the extracellular environment under drought stress conditions, suggesting that while the intracellular calcium stores provide Ca^{2+} to the cytoplasm, Ca^{2+} absorbed from extracellular environment was also an important way to accumulate calcium signaling ions in the pileorhiza. It is suggested that Ca^{2+}, as an important signaling molecule in response to drought stress, participated in the transmission of drought signaling to the roots of *P. edulis* seedlings.

3.3. Analysis of Ca^{2+} Signal Transport Patterns in the Pileorhiza of P. edulis under Drought Stress

The study of the Ca^{2+} signaling system in plant cells was mainly carried out by pretreatment of experimental materials with Ca^{2+} signal inhibitors [21]. Regulation of Ca^{2+} channels is vital. Plasma membrane Ca^{2+}-permeable channels interact with Ca^{2+} activated nicotinamide adenine dinucleotide phosphate (NADPH) to form a self-amplifying system—a ROS-Ca^{2+} hub [22]. This system could provide the transduction and amplification of the initial Ca^{2+} or reactive oxygen species (ROS) stimuli into a more sustainable response, with implications for cell growth, hormonal signaling, and stress response [23,24]. To obtain further evidence of the role of plasma membrane Ca^{2+} channels in *P. edulis* root tips, different Ca^{2+} inhibitors and ABA were used to further determine the Ca^{2+} signaling pathways. Ca^{2+} signal intensity varied with time in the pileorhiza of *P. edulis* seedlings treated with $LaCl_3$ (Ca^{2+} channel blocker), EGTA (extracellular Ca^{2+} chelating agent), and exogenous ABA.

To determine the factors affecting Ca^{2+} transport, Ca^{2+} flux in *P. edulis* root tip treated with a calcium antagonist and ABA was measured using NMT. As shown in Figure 6a, $LaCl_3$ was applied to the *P. edulis* seedlings treated with PEG-induced drought stress. The $LaCl_3$ treatment significantly

impeded extracellular Ca^{2+} influx at the pileorhiza compared to the experimental group without $LaCl_3$. Extracellular Ca^{2+} channels were involved in Ca^{2+} fluid transport. The extracellular Ca^{2+} in seedlings could enter cells through Ca^{2+} channels under drought stress, which was one of the reasons for the increase of cytoplasmic Ca^{2+} concentration.

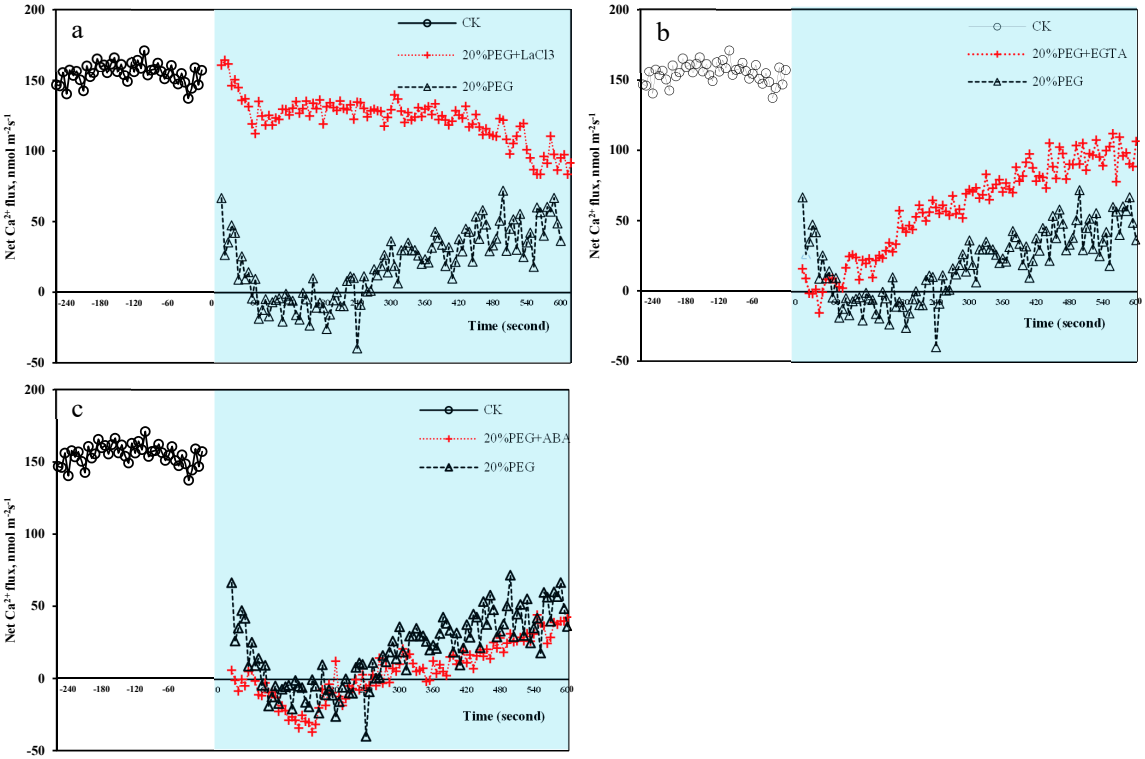

Figure 6. Factor analysis of Ca^{2+} flux of *P. edulis* seedlings under drought stress (Data Repetition: 3 replicates). The white area represents Ca^{2+} oscillation in the pileorhiza after treatment of the *P. edulis* seedlings with distilled water (CK), and the black line graph in the blue area indicates the Ca^{2+} oscillation in the pileorhiza treated with 20% PEG-induced drought stress for 10 min. The red line in the blue region indicates Ca^{2+} oscillation under 0.5 mmol/L $LaCl_3$ (**a**), 3 mmol/L EGTA (**b**), and 30 µmol/L ABA (**c**) in the 20% PEG treatment for 10 min.

In addition, EGTA was used to treat the roots of *P. edulis* seedlings under PEG-simulated drought stress. Ca^{2+} flux at the pileorhiza was almost always in a state of efflux (Figure 6b), while net Ca^{2+} flux of without EGTA treatment saw a pattern of "efflux-absorption-efflux". The EGTA-chelating extracellular Ca^{2+} increased, resulting in decreases of extracellular Ca^{2+} concentration and a decrease in the ability of pileorhiza to absorb Ca^{2+}; this caused an increase in Ca^{2+} excretion. Ca^{2+} uptake in the pileorhiza with the addition of EGTA was significantly lower than in the CK. It is worth noting that treating the pileorhiza with EGTA and $LaCl_3$ while under drought stress had the opposite effects on Ca^{2+} uptake curves. Ca^{2+} efflux rate from the pileorhiza of $LaCl_3$ decreased as EGTA treatment increased. $LaCl_3$ treatment hindered the Ca^{2+} channel in the pileorhiza, which led to a reduction of Ca^{2+} excretion from the pileorhiza.

Figure 6c shows Ca^{2+} absorption in the pileorhiza when treated with 30 µmol/L ABA for 10 min as PEG-induced drought stress treatment. Compared with the control, the ABA treatment showed a smaller oscillation in Ca^{2+} flux in the pileorhiza, and Ca^{2+} net flux reflected its strong absorption capacity. Therefore, ABA can promote the increase in Ca^{2+} absorption intensity in the pileorhiza of *P. edulis* seedlings. We conclude that ABA may activate the extracellular Ca^{2+} channel in the pileorhiza and promote Ca^{2+} uptake (Figure 6a,b). The efflux of ABA-induced Ca^{2+} spikes remained high among all treatments. These results further confirm that plasma membrane Ca^{2+} channels activity is involved

in Ca^{2+} signaling in PEG-induced drought stress by controlling (Ca^{2+})cyt through Ca^{2+} influxes. Under drought stress, adding the Ca^{2+} channel blocker $LaCl_3$ and extracellular Ca^{2+} chelating agent EGTA could significantly inhibit extracellular Ca^{2+} influx. Exogenous application of ABA could increase the ability of the pileorhiza to absorb Ca^{2+}.

3.4. Analysis of Ca^{2+} Signaling Pathway in Leaves under Drought Stress

In addition to chemical signal root transduction, drought stress triggered Ca^{2+} movement in leaf cells. Ca^{2+} may regulate leaf stomatal movement to control transpiration and respiration, as a self-protection and adaption measure in response to environmental stress. To simultaneously monitor cell-specific Ca^{2+} *P. edulis* seedling leaves, we used a confocal microscope to record Ca^{2+} fluorescence localization (Figure 7).

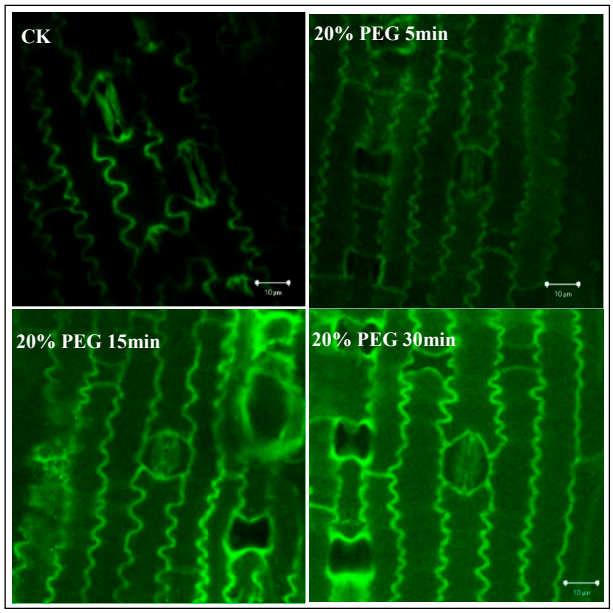

Figure 7. Localization of cellular Ca^{2+} dynamics in leaves of *P. edulis* seedlings. Calcium-fluorescence localization of the leaves of the *P. edulis* seedlings under drought stress for 5 min, 15 min, and 30 min induced by 20% PEG, respectively. The green fluorescence intensity represents the Ca^{2+} concentration and is positively correlated with the Ca^{2+} concentration.

CK is the Ca^{2+} fluorescence map of the lower epidermal cells treated with distilled water. Under normal water conditions, Ca^{2+} was mainly localized within the cell walls of guard cells, accessory cells, and long cells in *P. edulis* seedling leaves. Ca^{2+} was less commonly found to be distributed in the cytoplasm. As drought stress duration increased, Ca^{2+} concentration in the cytoplasm increased, except for in tethered cells. Among them, Ca^{2+} in long cells and guard cells increased significantly. The fluorescence intensity in the cytoplasm was also higher than that of the CK. Fluorescence intensity in the cytoplasm of accessory cells did not significantly increase. There were particularly pronounced differences in Ca^{2+} distribution in the 30 min PEG treatment. In addition to in tethered cells, the cytoplasmic fluorescence in all living cells, including guard cells, increased. Our results suggest that Ca^{2+} signals in response to PEG stress reflect distinct cellular Ca^{2+} dynamics.

With increased duration of drought stress, the fluorescence intensity in the cytoplasm of long cells increased. Only when a certain degree of drought stress was reached would Ca^{2+} in the cytoplasm of leaf guard cells escape from the cell wall and diffuse into the cytoplasm, resulting in a significant increase in the fluorescence intensity of the cytoplasm. In comparison, the Ca^{2+} in the cytoplasm of accessory cells was not obviously enhanced. To further verify that Ca^{2+} in the leaf cells of *P. edulis* was also transmitted through the cytoplasmic Ca^{2+} channels, three Ca^{2+} inhibitors were used to treat the

seedlings under drought conditions. Ca^{2+} fluorescence localization under these conditions is shown in Figure 8.

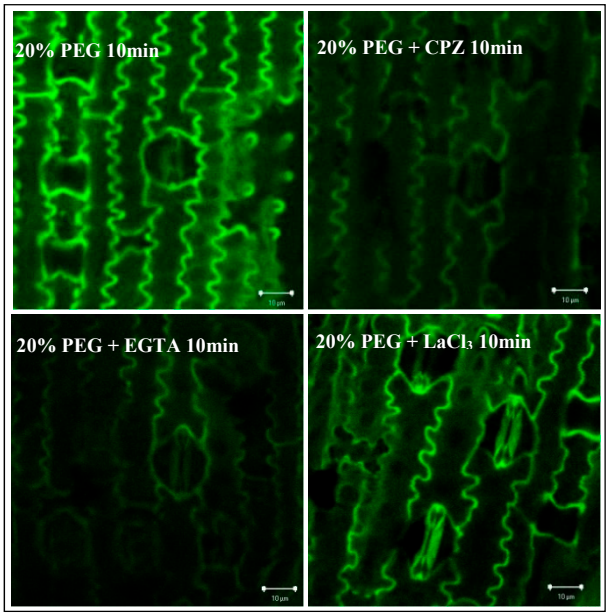

Figure 8. Localization of cellular Ca^{2+} dynamics in leaves of *P. edulis* seedlings treated with different Ca^{2+} inhibitors and drought stress. Ca^{2+} fluorescence localization in leaves of plants treated with 20% PEG-induced drought stress and 0.1 mmol/L CPZ, 3 mmol/L EGTA, or 0.5 mmol/L $LaCl_3$ for 10 min.

To confirm the effect of calcium channels on Ca^{2+}, we analyzed the Ca^{2+} fluorescence under three Ca^{2+} inhibitor treatments. The addition of CPZ inhibited the binding of Ca^{2+} to CAM in the seedlings; thus, the Ca^{2+} signal was not transmitted further. The results showed that after treatment with 0.1 mmol/L CPZ in the seedlings under PEG-simulated drought stress for 10 min, the brightness of the fluorescence in the cell wall and cytoplasm of the leaf cells was less than under the treatment without CPZ. Under drought stress, the Ca^{2+} in leaf cells of the *P. edulis* seedlings treated with 0.1 mmol/L CPZ were mainly distributed in the cell wall. The fluorescence brightness in the cell wall decreased more obviously than in other parts of the leaf.

We analyzed the distribution of Ca^{2+} in leaves treated with 3 mmol/L EGTA. Fluorescence intensity in the cell wall and cytoplasm reduced, indicating that Ca^{2+} concentration in the cell wall and cytoplasm decreased compared with the control group. This indicates that *P. edulis* seedling leaves can still take up Ca^{2+} from extracellular pathways under drought stress to produce calcium signals. Extracellular Ca^{2+} was chelated in the leaves under EGTA treatment, resulting in a decrease in extracellular Ca^{2+} uptake.

Treatment with $LaCl_3$ prevents Ca^{2+} from extracellular entry, and Ca^{2+} in the cytoplasm of leaf cells significantly reduced. Ca^{2+} was mainly concentrated in the cell walls in this treatment (Figure 8). The results of Ca^{2+} fluorescence localization in leaf cells after treatment with 0.5 mmol/L $LaCl_3$ for 10 min in drought-stressed *P. edulis* seedlings showed a decrease in Ca^{2+} fluorescence intensity in the cytoplasm of the cells. This was caused by the $LaCl_3$ blocking the cytoplasmic Ca^{2+} channel, blocking extracellular Ca^{2+} entry through the cell wall. This shows that Ca^{2+} in the leaves of *P. edulis* can be transported through Ca^{2+} channels of the plasma membrane, absorbing Ca^{2+} from outside the cell, and using the potential difference to generate calcium signals.

3.5. Analysis of the Effects of Ca^{2+} Signals on H_2O_2 and ABA Signaling Pathways under Drought Stress

The H_2O_2 fluorescence localization map of *P. edulis* leaves is shown in Figure 9. H_2O_2 concentration in the leaf cytoplasm increased with drought stress time (Figure 9a). *P. edulis* seedling

leaves treated with different Ca^{2+} inhibitors under PEG-simulated drought stress were subjected to laser confocal technology for H_2O_2 fluorescence localization to study the relationship between Ca^{2+} signaling and H_2O_2 in the stress signaling pathway (Figure 9b). Compared with CK, plants treated with 0.1 mmol/L CPZ showed lower leaf fluorescence, indicating that CPZ could also prevent H_2O_2 signal transduction. The calcium signals were unable to be transmitted normally, resulting in the decrease of H_2O_2 in the leaf cells. This indicates that the regulation of H_2O_2 activity requires the participation of Ca^{2+}. Both 0.5 mmol/L $LaCl_3$ and 3 mmol/L EGTA inhibited the production of H_2O_2 in guard cells, accessory cells, and long cells in leaves of *P. edulis* seedlings under drought stress. It is inferred that the Ca^{2+} signal is generated upstream of the active oxygen signal in the drought stress signaling network of *P. edulis*, and that H_2O_2 activity in leaf cells requires Ca^{2+} participation.

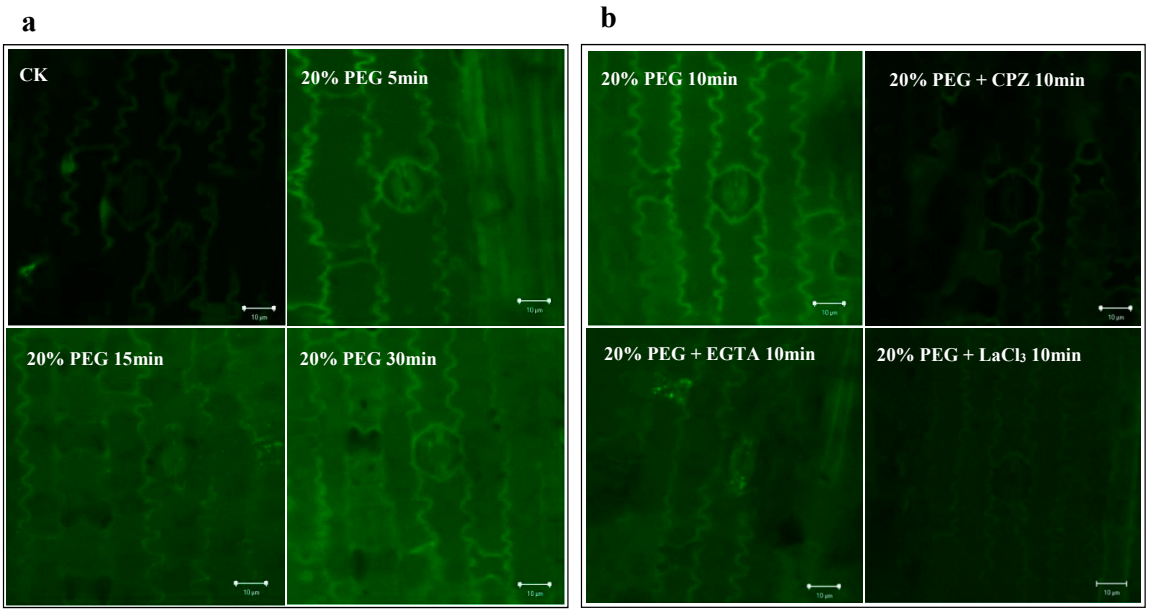

Figure 9. H_2O_2 fluorescence in *P. edulis* seedling leaves under drought stress. The green fluorescence intensity in the figure represents concentration of hydrogen peroxide, with more fluorescence indicating a higher concentration of H_2O_2. (**a**) H_2O_2 fluorescence localization at different treatment times (5 min, 15 min, and 30 min) using 20% PEG-induced drought stress. (**b**) The samples were treated with different Ca^{2+} inhibitors (CPZ, EGTA, and $LaCl_3$) for 10 min under 20% PEG-induced drought stress.

Laser confocal microscopy was used to detect the Ca^{2+} and H_2O_2 fluorescence signal in living leaf cells. The results indicated that the exogenous application of ABA under drought stress led to an increase in the concentration of Ca^{2+} and H_2O_2 in the mesophyll cells of *P. edulis* seedlings. Interestingly, there was a positive correlation between Ca^{2+} and H_2O_2 dynamics. ABA might activate Ca^{2+} channels of the plasma membrane and promote the production of Ca^{2+} signals in the pileorhiza of *P. edulis* seedlings.

H_2O_2 fluorescence intensity in guard cells was significantly higher in the ABA-treated group than in the group not treated with ABA (Figure 10). In plants, ABA is involved in many stress responses [25–27]. Regulatory systems of reactive oxygen species (ROS) are known to be integrated with other pathways involving Ca^{2+} signaling, protein kinases, and hormones pathways to regulate the defense mechanism in plants [28]. The existence of ABA-induced genes, which are expressed in stomatal guard cells, allows us to closely examine the role of Ca^{2+} [29]. In rice, the Ca^{2+}/CaM-dependent protein kinase OsDMI3 has been shown to be required for ABA-induced antioxidant defense [30]. These results demonstrated that ABA also plays a key role in the signal transduction of Ca^{2+} in the signaling network of the leaves of *P. edulis* treated with drought stress. Ca^{2+} not only acted as the upstream signal of H_2O_2, but was also involved in the signal transduction process of ABA. ABA could promote the production of Ca^{2+} signaling in leaves and stimulate the burst

of H_2O_2, a reactive oxygen species, in the guard cells. *P. edulis* may enhance drought tolerance via ABA-induced stomatal closure by ROS production.

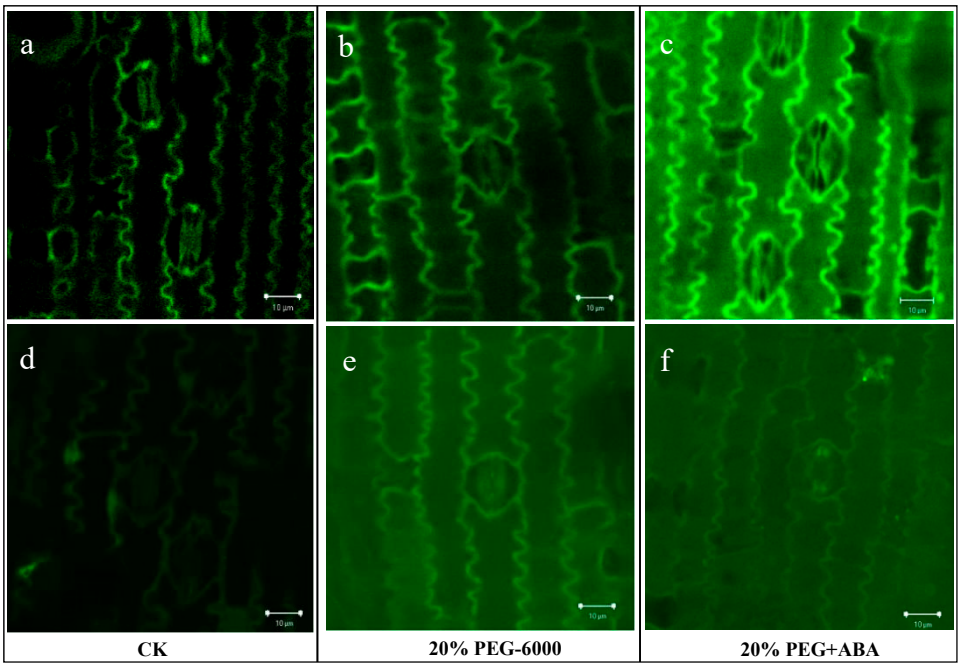

Figure 10. Localization of fluorescence of Ca^{2+} and H_2O_2 in mesophyll cells after application of exogenous ABA under drought stress. (**a**) Calcium-fluorescence localization of leaves of *P. edulis* seedlings with distilled water. (**b**) Calcium-fluorescence localization of leaves of *P. edulis* seedlings under 20% PEG 6000-induced stress for 10 min. (**c**) Calcium-fluorescence localization of leaves of *P. edulis* seedlings with the application of 30μmol/L ABA to 20% PEG-induced stress for 10 min. (**d**–**f**) H_2O_2 fluorescence localization under the same treatment as above.

4. Conclusions

This study indicated that the conductivity of *P. edulis* leaves increased with the degree of drought stress induced. Ca^{2+} is an important signaling molecule in response to drought stress in the root tip of *P. edulis*, especially in the transmission of stress signals and resistance to drought stress. Under drought stress, root tip cells of *P. edulis* can be used to transport Ca^{2+} from the extracellular environment to the cytoplasm, Ca^{2+} channels participate in extracellular Ca^{2+} transportation, and ABA may activate Ca^{2+} channels in the plasma membrane and promote Ca^{2+} signal production in the pileorhiza of *P. edulis*. In *P. edulis* leaves, Ca^{2+} can also be transported through the Ca^{2+} channels of the plasma membrane under drought stress, absorbing Ca^{2+} from outside the cell and generating Ca^{2+} signals through potential difference. The responsiveness of Ca^{2+} signals to drought stress in leaves of *P. edulis* from strong to weak was shown as follows: (1) long cells; (2) guard cells; (3) accessory cells; and (4) plug cells. Ca^{2+} acts as the upstream signal of H_2O_2 in the signal network of the mesophyll cells of the *P. edulis* under drought stress. It is also involved in ABA signal transduction process. ABA could promote Ca^{2+} signal production and stimulate H_2O_2 bursts in *P. edulis* leaves.

This study provides a method for the spatial and temporal localization of Ca^{2+} signaling and flux in *P. edulis.* Further research on Ca^{2+} signaling is essential, as it may help shed light on the physiology of *P. edulis* under stress. There are also areas for improvement in this study. The first is that processing the mesophyll samples may damage the cells or put them under stress conditions, thus affecting the Ca^{2+} fluorescence of the leaves under the laser confocal microscope. Autofluorescence of lignin can also have an effect on Ca^{2+} fluorescence. It is particularly important to improve this methodology in the future.

Author Contributions: X.J., C.C., and S.F. conceived and designed the experiments; X.J. performed the experiments; X.J. analyzed the data; L.W. and X.Z. contributed reagents/materials/analysis tools; X.J. wrote the paper, and C.C. revised it.

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Conflicts of Interest: The authors declare no conflict of interest.

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