Ca^{2+} Participates in Programmed Cell Death by Modulating ROS During Pollen Cryopreservation

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Research Article

Abstract

Programmed cell death (PCD) is one of the reasons for the decline in pollen viability after cryopreservation. However, the role of calcium ions (Ca^{2+}) in PCD during pollen cryopreservation has not been revealed in the existing studies. In this study, *Paeonia lactiflora* 'Fen Yu Nu' pollen was used as the research material for investigating the effects of Ca^{2+} changes on PCD indices and reactive oxygen species (ROS) during pollen cryopreservation. The results showed that after cryopreservation, with the decrease of pollen viability, the Ca^{2+} content significantly increased. The regulation of Ca^{2+} content had a significant effect on PCD indices, which showed that the Ca^{2+} carrier A23187 accelerated the decrease of mitochondrial membrane potential level and increased the activity of caspase-3-like and caspase-9-like proteases and the apoptosis rate. The expression levels of partial pro-PCD genes were upregulated, the anti-PCD gene *BI-1* was downregulated, and the addition of Ca^{2+} chelating agent EGTA had the opposite effect. The addition of the Ca^{2+} carrier A23187 after cryopreservation significantly increased the ROS content of pollen, the addition of the Ca^{2+} chelating agent EGTA had the opposite effect, and Ca^{2+} regulators also had significant effects on the contents of ROS production and clearance-related substances. Ca^{2+} affected intracellular ROS content by acting on the ROS production and clearance system during the cryopreservation of pollen and is thus involved in the occurrence of PCD.

Key Message

After cryopreservation, the Ca^{2+} content increased, which affected the intracellular ROS content, then participated in the occurrence of programmed cell death in pollen.

Introduction

Pollen, as an essential component of plant sexual reproduction, is an organic component comprised of 2–3 cells with highly differentiated functions that carries all of the genetic information of male gametophytes. Pollen can not only serve as a reference line for plant classification and variety origin and to study evolution, but it also is an important breeding material; in addition, due to its simple structure and consistent development, pollen can be used as a model material to study the signal transduction mechanism of plant cells and the polar movement of cells, which is an important part of plant research (Salmaki et al., 2008). However, in most cases, pollen has a relatively short life in vitro (Pio et al., 2007; Cruz et al., 2008), and some species or cultivars can only survive for hours or even minutes (Luna et al., 2001). Due to the importance of pollen in the field of plant research, the preservation of pollen has great significance.

Cryopreservation, as a biological technique, is one of the long-term, effective and safe methods for pollen preservation. However, pollen viability after cryopreservation shows a variety of trends, and in many cases, shows a significant decrease (Ren et al., 2019a). To date, multiple studies have shown that programmed cell death (PCD) is one of the key reasons for the pollen viability decrease. Jiang found that

the addition of catalase and pyruvate dehydrogenase to cryopreserved pollen of *Paeonia lactiflora* improved post-LN viability and inhibited PCD indices (Jiang et al., 2019a). Ren found that the addition of heat shock protein-70 at an appropriate concentration had certain inhibitory effects on the activity of caspase-3-like protease and the apoptosis rate, thus optimizing the pollen viability level of *Paeonia lactiflora* after LN storage (Ren et al., 2019b). In addition, in the pollen of six cultivars of *Paeonia suffruticosa*, which showed three change trends of viability after cryopreservation, PCD was found to be the key reason for the decreased viability (Ren et al., 2020a).

PCD is an active, orderly cell death process controlled by genes that involves a unique and complex signaling system and it is a normal physiological reaction of the organism in response to stress and its own metabolism (Kerr et al., 1972). In the study of plant cryopreservation, the occurrence of PCD is mainly caused by an increase of intracellular ROS level induced by the stress during the process of cryopreservation. This activates the permeability conversion channel of the mitochondrial membrane, leaded to the rapid release of cytochrome C by mitochondria, which activates downstream apoptotic proteases and initiates PCD, resulting in changes in viability after cryopreservation. Zhang, during the cryopreservation of embryogenic calli from *Agapanthus praecox*, showed that ROS-mediated activation of caspase-3-like protease was involved in the regulation of the occurrence of apoptosis-like PCD and thus had a certain influence on cell viability after cryopreservation (Zhang et al., 2015). Jiang, during the vitrification cryopreservation of protocorms of *Dendrobium nobile*, found that ROS were involved in the activation of caspase-3-like proteases and this mediated the occurrence of PCD, which was involved in the changes in cell activity before and after LN preservation (Jiang et al., 2019b). Similarly, in the cryopreservation of *Paeonia lactiflora* pollen, it was also confirmed that ROS-mediated PCD was one of the key reasons for the decreased viability after cryopreservation (Ren et al., 2020b).

Calcium ions (Ca^{2+}) are a major participant in biological and abiotic stress responses. Generally, the concentration of Ca^{2+} in plant cells is relatively low, but when the cells are stimulated by external environmental conditions, the intracellular Ca^{2+} content rapidly increases (Takezawa et al., 1995;Bush, 1995). A large number of studies have shown that increased Ca^{2+} is involved in the regulation of PCD in plants. Lin found that Ca^{2+} was an essential element to regulate PCD in protoplasts of *Nicotiana tobacum* induced by salt stress (Lin et al., 2005). Lachaud found that a high level of nuclear Ca^{2+} was an important mediator of PCD induced by d-erythro-sphinganine in tobacco cells (Lachaud et al., 2010). Additional studies showed that Ca^{2+} was involved in the regulation of PCD by regulating the changes in ROS content. In the process of chitosan-induced PCD in *Pisum sativum* cells, the addition of the Ca^{2+} chelating agent EGTA to the culture medium completely blocked the increase in Ca^{2+} content induced by chitosan and prevented the accumulation of intracellular ROS (Zuppini et al., 2003). During the infection of *Solanum lycopersicum* leaves with tobacco mosaic virus, high levels of Ca^{2+} in the cytoplasm participated in the regulation of PCD in root tip tissues by inducing an increase in ROS concentration (Li et al., 2018).

However, the role of Ca^{2+} in the occurrence of PCD and its possible pathways have not previously been reported in plant cryopreservation, and only the effect of cryopreservation on Ca^{2+} content has been reported. Zhang, in the cryopreservation of *Prunus mume* pollen, found that the Ca^{2+} content was significantly increased, which was speculated to be related to the stress response during the process of cryopreservation (Zhang, 2007). He found that the Ca^{2+} content in each organelle increased significantly during the vitrification cryopreservation of the embryogenic callus of *Agapanthus praecox* (He et al., 2014). Therefore, in this study, *P. lactiflora* 'Fen Yu Nu' pollen, with decreased viability after cryopreservation, was used to study the changes in Ca^{2+} content and its effects on PCD and ROS-related indicators during cryopreservation to explore the role of Ca^{2+} in the occurrence of PCD and its possible pathways during pollen cryopreservation.

Materials And Methods

Pollen collection and cryopreservation

Pollen of *P. lactiflora* 'Fen Yu Nu' was collected at the International Peony Garden in Luoyang of Henan Province (E 112°16- 112 ° , N 34°32- 34°45) on April 29, 2019. When the humidity level was appropriate, buds that were about to open were collected. Then, the anther was left for 24 h at room temperature (23±2 °C) to ensure complete pollen dispersion. Pollen was collected using an 80 mm aperture sieve (Ren et al., 2019a).

The collected pollen (0.1 g/package) was placed in a 2 mL cryopreserved tube, and then directly put into LN for storage. The pollen was thawed under running water (Thaw) or thawed at room temperature (LN).

Pollen moisture content and viability determination

The pollen moisture content was determined by the 105 °C constant temperature drying method.

The pollen germination rate was detected by the hanging drop culture method (Ren et al., 2019a). The medium was 10% sucrose and 0.1% boric acid. The culture was carried out at 25 °C for 4 h. The criterion for pollen germination was that the length of the pollen tube was greater than twice the diameter of the pollen grains. Four replicates were set for each treatment, and three fields were randomly selected for each replicate. The germination number was counted with a 20x eyepiece (Leica DM-500), and the mean value of the results was taken.

Pollen Ca^{2+} content determination

Fluo-3 AM (39294–11 ML, Sigma Chemical Co., St. Louis, MO, USA) fluorescence staining was used to determine the content of Ca^{2+} in the pollen (Zhang, 2007; Zhang et al., 2012). Approximately 0.01 g of pollen was added to 200 µL of Fluo-3 AM Ca^{2+} fluorescent probe (100 µmol/L) in the dark and thoroughly mixed. The pollen was incubated with the dye solution at a constant temperature of 25 °C for 30 min in the dark, followed by centrifugation at 2000 rpm for 20 s, and the dye solution was discarded. After

washing twice with 500 μL PBS (pH=7.4), 1 mL PBS (pH=7.4) was added, and the pollen was resuspended. The fluorescence value was measured in the FITC channel of a flow cytometer (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ, USA) without adding any fluorescent dye as a control. The actual fluorescence measurement value was taken as the relative content of Ca^{2+}. Each treatment was repeated 3 times, and the mean value of the results was taken as the relative content of Ca^{2+}.

Pollen mitochondrial membrane potential determination

The pollen mitochondrial membrane potential was detected by JC-1 fluorescence staining. Approximately 0.01 g pollen was added to 300 μL 4% paraformaldehyde and fixed at room temperature for 5 min. After that, 300 μL immunostaining permeabilization solution with Triton X-100 was added to the pollen cells for 5 min at room temperature. The permeable pollen cells were washed twice with 500 μL PBS (pH= 7.4).

An aliquot of 0.5 mL JC-1 staining solution (Biyuntian Biotechnology Co., Ltd., C2006) was added to the precipitated pollen cells, thoroughly mixed, and incubated for 30 min at 37 °C without light. The fluorescence staining was removed, and the stained pollen cells were washed twice with 500 μL of JC-1 staining buffer (1X). Then, 1 mL of JC-1 staining buffer (1X) was added to resuspend the fluorescently loaded pollen cells. Flow cytometry (BD-Facsaria Sorp, Becton-Dickinson, Franklin Lakes, NJ, USA) was used to detect the fluorescence values. Three replicates were set for each treatment. The negative control was pollen without fluorescence loading, and the red–green fluorescence ratio was taken as the result.

Pollen caspase-like protease activity determination

Caspase-3-like and caspase-9-like protease activities were detected using the kit provided by Biyuntian Biotechnology Co., Ltd. (C1116, C1158). Approximately 0.03 g pollen was ground into homogenate at low temperature with 300 μL lysis solution, and then placed in an ice bath for 5 min. The pollen was centrifuged at 12000 rpm at 4 °C for 20 min. Then, 50 μL extract was added to 40 μL test buffer, and 10 μL caspase-3-like protease reaction substrate Ac-DEVD-*p*NA (2 mM) or caspase-9-like protease reaction substrate Ac-LEHD-*p*NA (2 mM) was added to initiate the reaction. The cells were incubated in the dark at 37 °C for 2 h. Then, the absorbance value was measured by a spectrophotometer at 405 nm. Each treatment was repeated three times, and the average of the results was taken.

Pollen cells apoptosis rate determination

The apoptosis rate was detected by Annexin V-FITC and PI double staining. A weight of 0.01 g of pollen was taken for cell fixation and permeability treatment for mitochondrial membrane potential determination. Then, 200 μL cell staining buffer was added to a suspension of the treated pollen, and 10 μL Annexin V-FITC staining solution was added and incubated at 4 °C for 30 min without light. After Annexin V-FITC staining, 15 μL PI was added in the dark for 15 min. After that, the pollen wall was washed twice to remove the residual staining solution with 200 μL PBS (pH=7.4). Then, the precipitated pollen was resuspended in 600 μL PBS (pH=7.4), and the cells were filtered into a flow

measurement tube with an 80 μm aperture cell screen. The pollen suspension without any staining was used as the control, and the fluorescence values were measured by flow cytometry (BD-Facsariasorp, Becton-Dickinson, Franklin Lakes, NJ, USA). Each treatment was repeated three times.

qRT- PCR analysis of pollen PCD and ROS related genes

RNA was extracted using the Plant RNA Rapid Extraction Kit (RN38-EASYspin Plus) provided by Beijing Aidlai Biotechnology Co., Ltd., cDNA was synthesized using REVER TRA ACE ® qPCR RT Master Mix with GDNA Remover kit (TOYOBO, Osaka, Japan), and the primers were designed using IDT online software (https://sg.idtdna.com/Primerquest/Home/Index) and synthesized by Beijing Ruiboxingke Biological Technology Co., Ltd. (shown in Table S1 and Table S2).

The cDNA of *P. lactiflora* 'Fen Yu Nu' pollen was used as a template for PCR amplification, annealing temperature screening and primer specificity detection. The amplification system was 20 μL, including 1 μL cDNA, 2 μL forward primer (10 μM), 2 μL reverse primer (10 μM), 5 μL ddH$_2$O and 10 μL SYBR Premix Ex Taq (TAKARA, Otsu, Japan). The amplification protocols for qRT-PCR included an initial denaturing step (95 °C for 30 s), followed by 40 cycles of 95 °C for 10 s, T$_m$(°C) for 15 s, and 72 °C for 15 s; 65 °C for 5 s and 95 °C for 5 s (Wan et al., 2019). The expression level of the target gene was calculated by the $2^{-\Delta\Delta Cq}$ method, and each treatment was repeated 3 times.

Addition of Ca^{2+} and ROS exogenous regulator for pollen

Pollen preserved with LN thawed at room temperature (LN) was treated with different concentrations of Ca^{2+} carrier A23187 (21186- 10G, Sigma Chemical Co., St. Louis, MO, USA) and Ca^{2+} chelating agent EGTA (E3889- 10G, Sigma Chemical Co., St. Louis, MO, USA), 200 mmol/L ROS scavenger AsA (A7506-25G, Sigma Chemical Co., St Louis, MO, USA), 0.16 mmol/L ROS scavenger GSH (G6013-5G, Sigma Chemical Co., St Louis, MO, USA)or 200IU ROS enzyme scavenger CAT (C100-50MG, Sigma Chemical Co., St Louis, MO, USA) solutions at a solid-liquid ratio of 1:100 and thoroughly mixed. After incubation for 10 min in the dark at 37 °C, the regulator solution was removed by centrifugation at 2000 rpm for 30 s. Then, the pollen was washed three times with phosphate buffer of 0.01 M (PBS, pH= 7.4) and centrifuged at 2000 rpm for 30 s. The precipitated pollen was used for the determination of the various indices.

Pollen ROS content determination

The ROS content of the pollen was determined by DCFH-DA (D6883–50MG, Sigma Chemical Co., St Louis, MO, USA) fluorescence staining (Xu et al., 2014). Then, 0.01 g of pollen was added to 200 μL of DCFH-DA staining solution (100 μmol/L) under dark conditions and thoroughly mixed. The pollen was incubated at 37 °C for 30 min without light and centrifuged at 2000 rpm for 20 s, and then the dye solution was discarded. After washing twice with 500 μL PBS (pH= 7.4), 1 mL PBS (pH= 7.4) was added, and the pollen was resuspended. Flow cytometry (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ, USA) was used to measure the fluorescence value in the FITC channel. The relative content of ROS was taken as the actual fluorescence measurement value, and each treatment was repeated 3 times.

Pollen NDAPH oxidase activity determination

Nicotinamide adenine dinucleotide phosphate (NDAPH) enzyme activity was determined according to the NADP+/NADPH detection kit (S0179) provided by Biyuntian Biotechnology Co., Ltd. Pollen (0.01 g) was homogenated in an ice bath with 400 μL NADP$^+$/NADPH extract, and centrifuged at 12,000 rpm for 10 min at 4°C. Added 200 μL G6PDH working solution into 100 μL extraction solution, incubated for 10 min at 37°C without light. Then add 10 μL chromogen solution, mixed well and incubated at 37°C for 60 min without light. The absorbance was measured at 450 nm with an ultraviolet spectrophotometer (Thermo Scientific GENESYS-150), each treatment was repeated three times, and the results were averaged.

Pollen antioxidant enzymes and antioxidants determination

Superoxide dismutase (SOD) determined by nitrogen blue tetrazole (NBT) reduction method (Li et al., 2000). Pollen (0.05 g) was homogenated with 1 mL phosphoric acid buffer (0.05 mol/L) in an ice bath, and centrifuged at 10,000 rpm for 15 min at 4°C. Added 0.05 mol/L phosphoric acid buffer (1.5 mL), 130 mmol/L methionine solution (1.5 mL), 750 mol/L NBT solution (300 μL), 100 mol/L EDTA-Na$_2$ (300 μL) and 20 μmol/L riboflavin solution (300 μL) into 300 μL extract solution in the dark. Mixed and placed in the light condition (4000 Lx) reaction for 15 min, and terminated the reaction in darkness. Measured the absorbance value at the wavelength of 560 nm. Each treatment was performed on triplicate, and the results were averaged.

Catalase (CAT) was measured according to the Prochakova method and with slight modifications (Prochazkova *et al.*, 2001). Ground 0.05 g pollen into homogenate with 1 mL phosphate buffer (0.05mol/L) in an ice bath, and centrifuged at 10,000 rpm for 15 min at 4°C. Added 1.5 mL phosphoric acid buffer (pH= 7.0) and 1.0 mL distilled water into 200 μL extract solution. Mixed and initiated the reaction with 300 μL hydrogen peroxide solution. Measured the absorbance at 240nm. Each treatment was repeated three times, and the average of the results was taken.

Glutamate reductase (GR) activity was determined by the kit (S0055, Biyuntian Biotechnology Co., Ltd.). One mL phosphate buffer solution (0.01 mol/L) was used to grind 0.03 g pollen to homogenate in an ice bath, and centrifuged at 10,000 rpm for 15min at 4°C. The supernatant was kept as the enzyme solution for the determination of GR activity. Each treatment was performed on triplicate, and the results were averaged.

Ascorbic acid peroxidase (APX) activity was measured according to the Nakano and Asada method and with slight modification (Nakano and Asada, 1981). Ground 0.03 g pollen with 1 mL APX enzyme extraction reagent (50 mmol/L PBS+2 mmol/L AsA+5 mmol/L EDTA) to homogenate in an ice bath, and centrifuged at 10,000 rpm for 20 min at 4°C. Added 2 mL PBS (50 mmol/L, pH=7.0), 500 μL AsA solution (5 mmol/L) and 500 μL EDTA-Na$_2$ (1 mmol/L) into 50 μL supernatant extract. Then initiated the reaction with 50 μL H$_2$O$_2$ (30%), the absorbance at 290 nm was determined by a spectrophotometer (Thermo Scientific GENESYS-150). Each treatment was repeated three times, and the average of the results was taken.

Ascorbic acid (AsA) content was determined by Kampfenkel's method and with some modifications (Kampfenkel *et al.*, 1995). Pollen (0.03 g) was homogenated with 1.5 mL trichloroacetic acid (10%) in an ice bath, and the centrifuged at 10,000 rpm for 10 min at 4°C. In 200 μL supernatant, successively added 200 μL sodium dihydrogen phosphate solution (150 mmol/L) and 200 μL ddH$_2$O into, mixed at least 30 s. Then successively added 10% trichloroacetic acid solution, 44% phosphoric acid solution and 4% 2, 2-dipyridine solution, 400 μL each. Mixed and initiated the reaction by 200 μL FeCl$_3$ (3%), then placed at 37°C for 60 min. The absorbance value was determined by a spectrophotometer (Thermo Scientific GENESYS-150) at 525 nm, each treatment was performed on triplicate, the mean value was taken.

Glutathione (GSH) content was determined by 2-nitrobenzoic acid (DTNB) reduction method (Griffith, 1980). Ground 0.03 g pollen into homogenate with 1.5 mL trichloroacetic acid (10%) in an ice bath, and centrifuged at 10,000 rpm for 10 min at 4°C. In 0.5 mL supernatant, added 0.5 mL ddH$_2$O, 1 mL phosphate buffer (1 mmol/L, pH= 7.7) and 0.5 mL DTNB (4 mmol/L), mixed well and reacted at 25°C for 10 min. The absorbance values were measured at 412 nm, each treatment was performed on triplicate, and the results were averaged.

Statistical analysis

Flow cytometry data were processed and analyzed with FlowJo software. SPSS 17.0 (Version 17.0 SPSS Inc., Chicago, IL, USA) was used for one-way ANOVA analysis. Microsoft Excel 2013 software (Microsoft Corp., Richmond, CA, USA) was used for chart and table preparation.

Results

Moisture content and viability changes before and after cryopreservation of pollen

The moisture content of the pollen of *P. lactiflora* 'Fen Yu Nu' was 8.10%, and the viability of the fresh pollen was 22.73%. However, the viability of the pollen decreased after cryopreservation (Tab. 1). Compared with fresh pollen, the pollen viability after thawing at room temperature and after thawing with running water treatment after LN storage decreased by 6.31% and 1.93%, respectively.

Table 1
Moisture content and germination percentage of *P. lactiflora* pollen

Cultivar	Moisture content / %	Germination percentage / %		
		Fresh(CK)	LN	Thaw
P. lactiflora 'Fen Yu Nu'	8.10±0.59	22.73±1.64a	16.42±0.82b	20.80±2.27a

Changes in the Ca $^{2+}$ content of the pollen before and after cryopreservation

The Ca^{2+} content of *P. lactiflora* 'Fen Yu Nu' was significantly changed after cryopreservation (Fig. 1). The Ca^{2+} contents of the pollen thawed at room temperature and thawed with running water after preservation with LN were all significantly higher than that of fresh pollen (P< 0.05), increased by 84.94% and 83.12%, respectively.

Changes in the Ca $^{2+}$ content of pollen with and without Ca^{2+} regulator treatment

The addition of Ca^{2+} regulators after cryopreservation had a certain regulatory effect on the endogenous Ca^{2+} content of pollen (P < 0.05). The addition of different concentrations of the Ca^{2+} carrier A23187 significantly increased the endogenous Ca^{2+} content of pollen after LN storage, especially the effect of 100 µM A23187 (Fig. 2- C2, I). The Ca^{2+}-chelating agent EGTA had a significant inhibitory effect on endogenous Ca^{2+} content only when the concentration was up to 1 mM (Fig. 2- H2, J).

Changes in PCD indicators of pollen with and without Ca $^{2+}$ regulator treatment

After preservation in LN, the addition of an appropriate concentration (100 µM) of the Ca^{2+} carrier A23187 accelerated the transformation of pollen JC-1 from polymer to monomer and significantly reduced the ratio of polymer to monomer (P < 0.05), indicating a significant decrease in the mitochondrial membrane potential level (Fig. 3- A2, B). The addition of the Ca^{2+} chelating agent EGTA (1 mM) had little effect on the presence of JC-1 in the mitochondrial matrix (Fig. 3- A3); although the ratio of polymers to monomers was higher than that of the control, the difference was not statistically significant (Fig. 3- B).

After preservation with LN, the addition of Ca^{2+} regulators had a significant effect on caspase-like proteases in the pollen (P< 0.05). The Ca^{2+} carrier A23187 (100 µM) significantly promoted the activation of caspase-3-like protease and caspase-9-like protease, and the addition of the Ca^{2+} chelating agent EGTA (1 mM) significantly inhibited the activity of these two proteases (Fig. 4).

After LN storage, the addition of 100 µM Ca^{2+} carrier A23187 significantly increased the apoptotic rate of pollen (P < 0.05) by 52.94% compared with the control (Fig. 5- B, D), while the addition of Ca^{2+} chelating agent EGTA (1 mM) significantly inhibited the apoptosis rate by 23.53% compared with the control (Fig. 5- C, D).

After preservation with LN, the Ca^{2+} carrier A23187 at an adaptive concentration (100 µM) significantly upregulated the expression levels of the pro-PCD genes *PDCD4*, *PDCD2*, *SBT3* and downregulated the expression levels of the anti-PCD genes *DADI*, *BI-1*, *HXK1* and *LSD1*, while the addition of the Ca^{2+} chelating agent EGTA (1 mM) after preservation in LN significantly downregulated the expression levels of the pro-PCD genes *PDCD4*, *PDCD2*, *SBT3*, *ATG8CL*, *BAG6*, *RTNLB2* and upregulated the expression levels of the anti-PCD gene *BI-1* (Fig. 6).

Changes in the ROS content of pollen with and without Ca $^{2+}$ regulator treatment

After cryopreservation, regulated Ca^{2+} had a significant effect on the endogenous ROS content (P < 0.05). Adding an appropriate concentration of the Ca^{2+} carrier A23187 (100 μM) significantly increased the intracellular ROS content of pollen (P < 0.05), while 1 mM Ca^{2+} chelating agent (EGTA) significantly inhibited endogenous ROS accumulation (Fig. 7-D).

Changes in regulatory genes related to ROS production and elimination substances in pollen with and without Ca $^{2+}$ regulator treatment

After LN storage, the addition of Ca^{2+} carrier A23187 at an appropriate concentration (100 μM) significantly upregulated the expression levels of *RBOHJ* (involved in ROS production by regulating NADPH enzymes) and *SODA* and downregulated the expression levels of *APX3*, *DHAR2*, *GSTU8*, *GPX6* and *GSH2*, while the addition of Ca^{2+} chelating agent EGTA (1 mM) significantly downregulated the expression levels of *RBOHJ* and *DHAR2* and significantly upregulated the expression levels of *APX3*, *GSTU8*, *GPX6* and *GSH2* but had no effect on *SODA* and *CAT1* (Fig. 8).

Changes in ROS production and elimination substances in pollen with and without Ca $^{2+}$ regulator treatment

The addition of Ca^{2+} regulators had a significant effect on the activity of NADPH enzymes after cryopreservation (Fig. 9). Adding 100 μM Ca^{2+} carrier A23187 significantly promoted the activation of NADPH enzyme (P < 0.05), which was increased by 68.22% compared with the control; however, adding 1 mM Ca^{2+} chelating agent EGTA significantly inhibited the enzymatic activity of NADPH.

After cryopreservation, the addition of Ca^{2+} carrier A23187 (100 μM) significantly decreased CAT activity, APX activity, and the ASA and GSH content but significantly increased the SOD activity, while the addition of Ca^{2+} chelating agent EGTA (1 mM) significantly promoted the activation of CAT and APX and accelerated the accumulation of ASA and GSH content (Fig. 10- B, C, E, F). However, the addition of Ca^{2+} carriers and chelating agents had no significant effect on GR activity (Fig. 10-D).

Changes in PCD related genes of pollen with and without ROS regulator treatment

After preservation with LN, the ROS scavenger AsA at an adaptive concentration (200 μM) significantly downregulated the expression levels of the pro-PCD genes *PDCD4*, *PDCD2*, *SBT3*, *ATG8CL*, *ACD11*, *BAG6*, *RTNLB2*, *AMC9* and upregulated the expression levels of the anti-PCD genes *DADI*, *BI-1*, *LSD1*. Pollen supplemented with 0.16 mM GSH after cryopreservation significantly downregulated the expression levels of the pro-PCD genes *PDCD4*, *PDCD2*, *ATG8CL*, *BAG6*, *RTNLB2* and upregulated the expression levels of the anti-PCD gene *BI-1*. While the addition of the ROS enzyme scavenger CAT (200 IU) after preservation in LN significantly downregulated the expression levels of the pro-PCD genes *PDCD4*, *BAG6*, *RTNLB2* and upregulated the expression levels of the anti-PCD gene *DADI* (Fig. 11).

Discussion

As an important intracellular signaling molecule, Ca^{2+} plays an important role in the plant response to various stress conditions (White et al., 2003; Choi et al., 2014; Gilroy et al., 2014). In this study, the Ca^{2+} content of *P. lactiflora* 'Fen Yu Nu' pollen was significantly increased after LN storage (Fig. 1). This is consistent with Zhang's findings of an increase in pollen Ca^{2+} content due to LN cryopreservation of several cultivars of *Prunus mume* (Zhang, 2007). This result indicated that the stress involved in cryopreservation increased the intracellular Ca^{2+} concentration of the pollen.

As intracellular Ca^{2+} concentrations increase, a range of physiological and developmental phenomena are triggered (Nazir et al., 2020). Increasing numbers of studies have found that a high level of Ca^{2+} is an important mediating molecule that triggers various kinds of PCD in plants. The cytoplasmic Ca^{2+} content was significantly increased in *Nicotiana tobacum* during PCD induced by salt stress (Lin et al., 2005). In the process of tobacco mosaic virus (TMV) infection of *Solanum lycopersicum* leaves, which causes systemic PCD in root tip tissue, high levels of Ca^{2+} play an important role in the regulation of plant PCD (Li et al., 2018). Ca^{2+} levels rapidly increased during high-temperature stress-induced PCD in suspension culture cells of *Arabidopsis thaliana* (Kacprzyk et al., 2017). In this study, as the pollen viability decreased, the Ca^{2+} content increased significantly (Table 1, Fig. 1). Studies have shown that PCD is the key reason for the decline in pollen viability after cryopreservation (Ren et al., 2020a; Ren et al., 2020b). Thus, the change in Ca^{2+} during the cryopreservation of *Paeonia lactiflora* pollen may also be one of the influencing factors of PCD, which shows that the relationship between Ca^{2+} and PCD may be similar to that observed during plant stress responses.

Adding the appropriate Ca^{2+} carrier A23187 to *P. lactiflora* 'Fen Yu Nu' pollen after cryopreservation not only promoted the accumulation of endogenous Ca^{2+} but also increased the activity of caspase-3-like protease and caspase-9-like protease and decreased the level of the mitochondrial membrane potential, thus increasing the rate of cell apoptosis and decreasing the rate of living cells (Fig. 2- 5). As a common exogenous Ca^{2+} carrier, A23187 not only promotes Ca^{2+} passing through the outer membrane but also promotes Ca^{2+} passing through the intima, thus increasing the intracellular accumulation of Ca^{2+} (Óbrien and Ferguson, 1997). A large number of studies have shown that A23187 accelerates the occurrence of PCD by increasing the intracellular Ca^{2+} content. The exogenous Ca^{2+} carrier A23187 can stimulate PCD in *Nicotiana tobacum* cell cultures (Óbrien and Ferguson, 1997). During PCD regulation of leaf perforation formation in *Aponogeton madagascariensis*, the exogenous Ca^{2+} carrier A23187 accelerated the occurrence of PCD and thus increased the rate of perforation formation (Fraser et al., 2020). In addition, this study found that the endogenous Ca^{2+} content of *P. lactiflora* 'Fen Yu Nu' pollen was significantly reduced when the appropriate concentration of Ca^{2+} chelating agent EGTA was applied to the pollen after LN storage, and the activity of caspase-3-like and caspase-9-like protease, the rate of apoptosis and living cells were inhibited to a certain extent with EGTA treatment, but there was no significant effect on the mitochondrial membrane potential (Fig. 2- 5). Previous studies have shown that EGTA, as a Ca^{2+}-specific chelator, can inhibit the occurrence of PCD to some extent. In the process of chitosan-induced PCD in *Pisum sativum* cells, Ca^{2+} transients were completely blocked by the addition of EGTA at an appropriate

concentration to the medium, and downstream reactions such as extracellular H_2O_2 accumulation and the activation of caspase-3-like proteases in early and late PCD were prevented (Zuppini et al., 2003). The Ca^{2+} chelating agent EGTA significantly blocked the increase in Ca^{2+} content and delayed the occurrence of PCD in *N. tobacum* protoplasts induced by salt stress (Lin et al., 2005). These results indicate that Ca^{2+} is positively involved in the regulation of PCD during pollen cryopreservation. And this results also suggests that the amount of chelating agent EGTA may need to be tried very carefully and that low doses of Ca^{2+} may help pollen survive after freezing in LN.

Moreover, at the gene level, it was found in this study that the addition of the appropriate concentration of Ca^{2+} carrier A23187 after LN preservation upregulated the expression levels of the pro-PCD genes *PDCD2*, *PDCD4*, *SBT3* and downregulated the expression levels of the anti-PCD gene *BI-1*. However, EGTA with an appropriate concentration of Ca^{2+} chelator downregulated the expression levels of the pro-PCD genes *PDCD2*, *PDCD4*, *SBT3* and significantly upregulated the expression levels of the anti-PCD gene *BI-1* (Fig. 6). *PDCD* regulates different types of PCD mainly by binding to histone acetyltransferases, which trigger the release of cytochrome C, activate caspase-3 protease, and translocate the pro-apoptotic factor *Bax* from the cytoplasm to the mitochondria (Chen et al., 2001;Chen et al., 2006). As a *Bax* homologous suppressor gene, *BI-1* is a PCD suppressor gene located in the endoplasmic reticulum (Kawei-Yamada et al., 2001), and studies in plants have shown that the interaction of the *BI-1* gene with *Bax* may determine the occurrence of early PCD in cells under cold stimulation (Zhao et al., 2014). These results indicate that Ca^{2+} not only participates in the regulation of PCD in the mitochondrial pathway but also participates in the regulation of PCD through the endoplasmic reticulum pathway during pollen cryopreservation. This is consistent with the results of previous studies; Ca^{2+} has multiple contact points in mitochondria and endoplasmic reticulum apoptosis pathways, and the two apoptotic pathways communicate through Ca^{2+} transport (Men et al., 2012). However, its specific role in pollen cryopreservation remains to be further explored.

ROS, as the key molecules involved in the mitochondrial and endoplasmic reticulum pathways of PCD, can activate the downstream apoptotic enzyme caspase-3 by regulating the release of cytochrome C, thus initiating PCD (Reape and McCabe, 2010; Reape et al., 2015). In this study, the Ca^{2+} regulator not only affected PCD but also affected the endogenous ROS content. The appropriate concentration of the Ca^{2+} carrier A23187 significantly increased the endogenous ROS content, while the appropriate concentration of the Ca^{2+} chelating agent EGTA significantly reduced the accumulation of endogenous ROS (Fig. 7). These results indicated that the change in Ca^{2+} concentration has a positive regulatory effect on ROS during pollen cryopreservation, which is consistent with a large number of existing research studies. During the process of chitosan-induced PCD in *Pisum sativum* cells, the addition of the Ca^{2+} chelating agent EGTA to the culture medium completely blocked the Ca^{2+} transients induced by chitosan and prevented intracellular ROS accumulation (Zuppini et al., 2003). In the process of PCD in root tip tissue caused by tobacco mosaic virus infection of *S. lycopersicum* leaves, high levels of Ca^{2+} in the cytoplasm can result in an increase in intracellular ROS concentration (Li et al., 2018).

In this study, the expression of most PCD related genes was consistent with the addition of Ca^{2+} chelating agent and ROS scavengers; especially the expression of pro-PCD genes *PDCD4*, *BAG6* and *RTNLB2* showed high consistent changes in Ca^{2+} inhibition and ROS clearance, and in the removal of Ca^{2+}, the inhibition of this three genes is better than ROS scavengers (Fig. 6, 11). *BAG6*, a member of the *Bcl-2* gene family, which is a key gene involved in regulating the occurrence of PCD (Li at al., 2016); *RTNLB2* mainly interacts with *Bcl-2* to reduce its transport level from endoplasmic reticulum to mitochondria, resulting in reduced anti-apoptotic activity of *Bcl-2*, thus exacerbating the occurrence of PCD (Nziengui and Schoefs, 2009); *PDCD* gene is involved in the occurrence of PCD mainly through the transport of proapoptotic factor *Bax* from cytoplasm to mitochondria (Chen et al., 2001; Chen at al., 2006). These results indicate that Ca^{2+} is involved in the occurrence of PCD by acting on ROS in pollen cryopreservation. In addition, the effect of Ca^{2+} on ROS not only involved in the mitochondrial pathway PCD, but also endoplasmic reticulum pathway PCD, and may also be an intermediary molecule of substance exchange between the two pathways. But the mechanism of it remains to be further discussed.

The changes in ROS content are mainly due to the balance between its production and scavenging systems. Among them, ROS production is mainly dependent on complex Ⅰ and complex Ⅲ of the mitochondrial electron transport chain as well as NADPH oxidase located in the plasma membrane (Huang et al., 2016; Noctor et al., 2007). In animals, mitochondria-plasma membrane-bound NADPH oxidase is a major source of ROS and it plays a central role in inducing cell death (Dan Dunn et al., 2015). In plants, NADPH oxidase on the plasma membrane is also an important ROS production site (Foyer and Noctor, 2005;Li et al., 2007；Monetti et al., 2014), and it is an important enzyme that produces most ROS signals (Apel and Hirt, 2004；Potocký et al., 2007；Cárdenas et al., 2008). Previous studies have shown that Ca^{2+} has a regulatory effect on the activity of NADPH oxidase and thus has a regulatory effect on ROS production (Torres et al., 1998). This is mainly because NADPH oxidase has an EF-hand domain that can bind to Ca^{2+} (Sagi and Fluhr, 2001; Bánfi et al., 2004), and Ca^{2+} has a regulatory effect on NADPH oxidase activity, so it has a regulatory effect on ROS production (Torres et al., 1998). In this study, the appropriate concentration of the Ca^{2+} carrier A23187 significantly increased the NADPH oxidase activity of pollen after LN storage and had a positive regulatory effect on the expression level of the NADPH oxidase regulatory gene *RBOHJ*, while the exogenous Ca^{2+} chelating agent EGTA significantly inhibited the activity of the NADPH enzyme and downregulated the expression of its related gene *RBOHJ* after cryopreservation (Fig. 8- 9). This is consistent with the results of previous studies. In the early stage of salt stress-induced PCD in *Oryza sativa* root tip cells, Li found that it inhibited the activity of NADPH oxidase and the accumulation of ROS by blocking Ca^{2+} inflow, thus slowing down PCD (Li et al., 2007). In conclusion, plasma membrane NADPH oxidase is one of the key sites for Ca^{2+} to produce ROS in cryopreserved pollen.

In addition, previous studies have shown that changes in the Ca^{2+} concentration also have certain effects on enzymes and nonenzymatic antioxidant substances involved in ROS scavenging. Liu applied Ca^{2+}

regulatory agents to the root of *Oryza sativa*, which significantly affected the activities of CAT and peroxidase (POD) and thus had a regulatory effect on intracellular ROS levels (Liu et al., 2016). In the early stage of salt stress-induced PCD in root tip cells of *Oryza sativa*, Li found that application of the Ca^{2+} production regulator La^{3+} prevented the increase in cytoplasmic Ca^{2+} levels and produced significant inhibitory effects on ROS scavenging, thus inhibiting ROS production (Li et al., 2007). In this study, the addition of the Ca^{2+} carrier A23187 had certain inhibitory effects on the activities of CAT and APX, as well as on the contents of ASA and GSH in pollen cells after LN storage, and it significantly downregulated the expression levels of the ROS scavenging regulatory genes *APX3*, *GSTU8*, *GPX6* and *GSH2*, while the Ca^{2+} chelating agent EGTA significantly promoted CAT and APX activities, the ASA and GSH contents, and significantly upregulated the expression of *APX3*, *GSTU8*, *GPX6* and *GSH2* (Fig. 8, 10). These results indicated that Ca^{2+} plays a role in regulating the level of endogenous ROS in cryopreserved pollen by acting on CAT activity and the content of substrates related to the ASA-GSH circulation system.

Conclusion

This study explored the mechanism of Ca^{2+} in PCD during pollen cryopreservation. During pollen cryopreservation, Ca^{2+} regulated the changes of endogenous ROS content by acting on the balance between intracellular ROS production and scavenging system, especially NADPH oxidase and some reaction substrates involved in the ASA-GSH antioxidant cycle system, and the effects involved the gene level, thus regulated the occurence of PCD in pollen. The pollen viability of *P. lactiflora* 'Fen Yu Nu' decreased significantly with increasing Ca^{2+} content after cryopreservation, and the regulation of Ca^{2+} not only significantly affected the PCD events but also affected the content of endogenous ROS and its production and scavenging-related substances.

Declarations

Conflict of interest: The authors declare that they have no conflict of interest.

Author contribution statement: Ruifen Ren designed the research, completed the experiments，analyzed the data and drafted the manuscript. Hao Zhou, Lingling Zhang offered some help on the material collection and technical aspects of the experiment. Xueru Jiang offered some help on the research design. Yan Liu conceived the project, supervised the analysis and critically revised the manuscript. All authors read and approved the manuscript.

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Figures
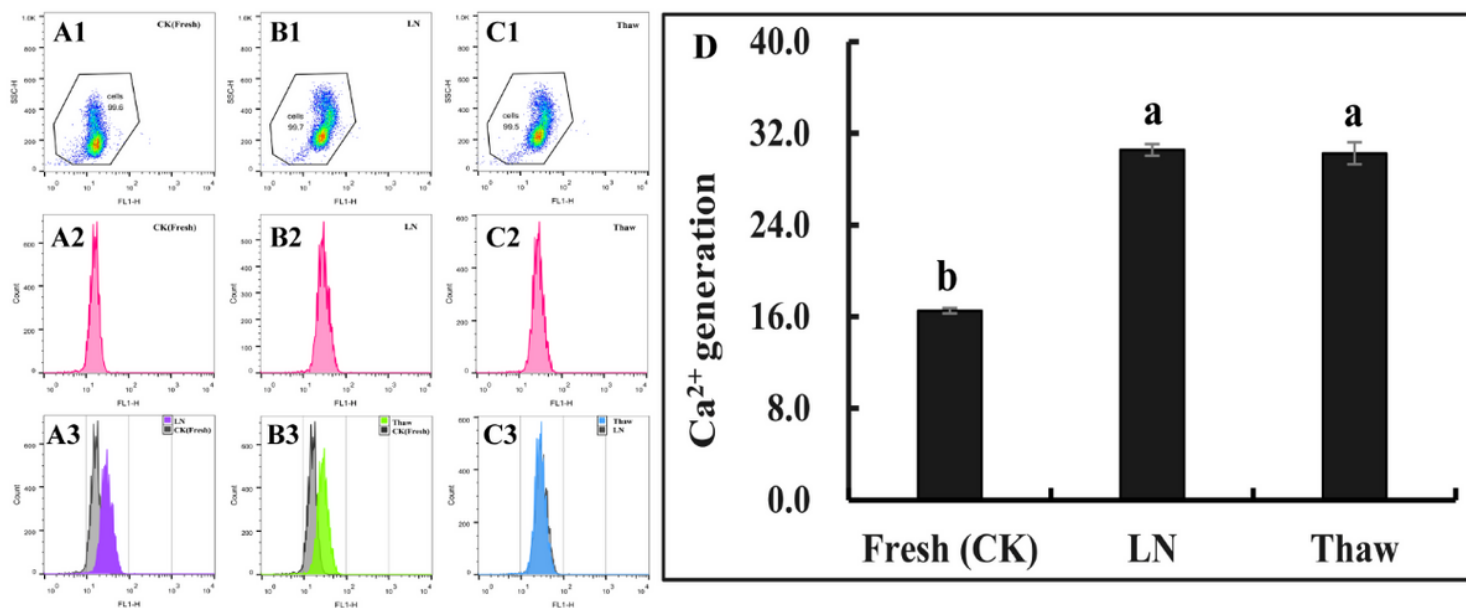
Figure 1

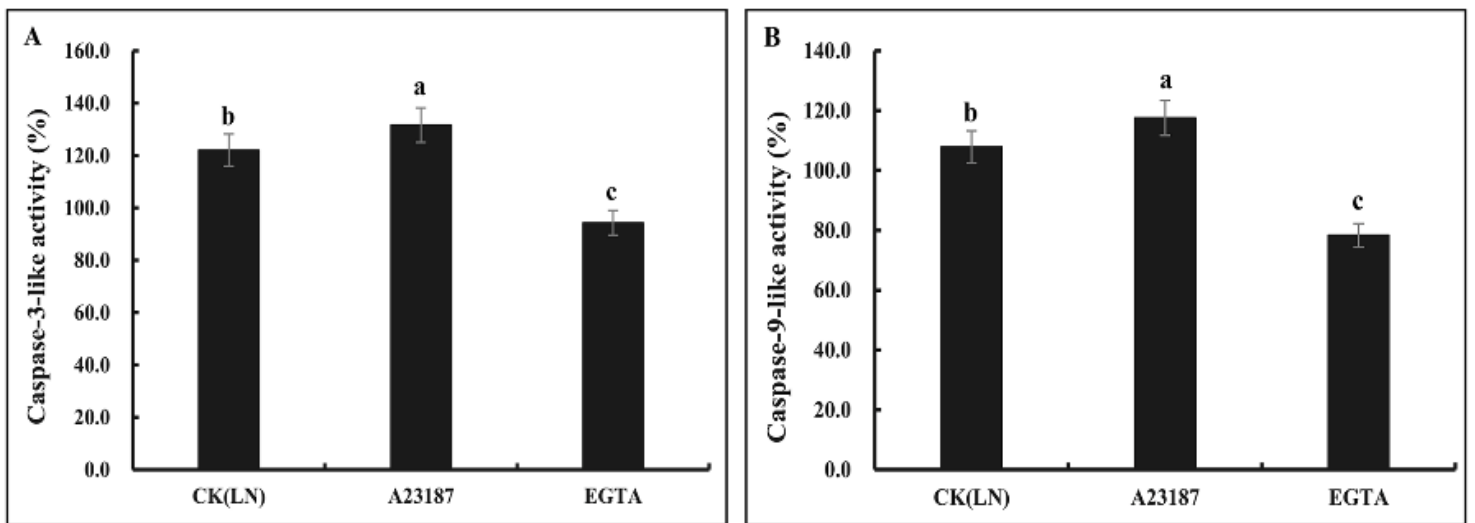Changes of Ca^{2+} content in pollen before and after cryopreservation.

Notes: Different letters indicate significant differences among different treatment at the 0.05 level (P< 0.05, Duncan's multiple range test). A- fresh pollen (CK); B- LN preserved pollen without running water thaw treatment (LN); C- the LN preserved pollen with running water thaw treatment (Thaw); D- the Ca^{2+} content of pollen; 1- the flow cytometry maps; 2- the flow spectrogram of Fluo-3 AM fluorescence (Ca^{2+} fluorescent probe); 3- the comparison spectrograms. Fresh (CK): fresh pollen without cryopreservation; LN: pollen thawed at room temperature after cryopreservation; Thaw: pollen thawed with running water after cryopreservation.
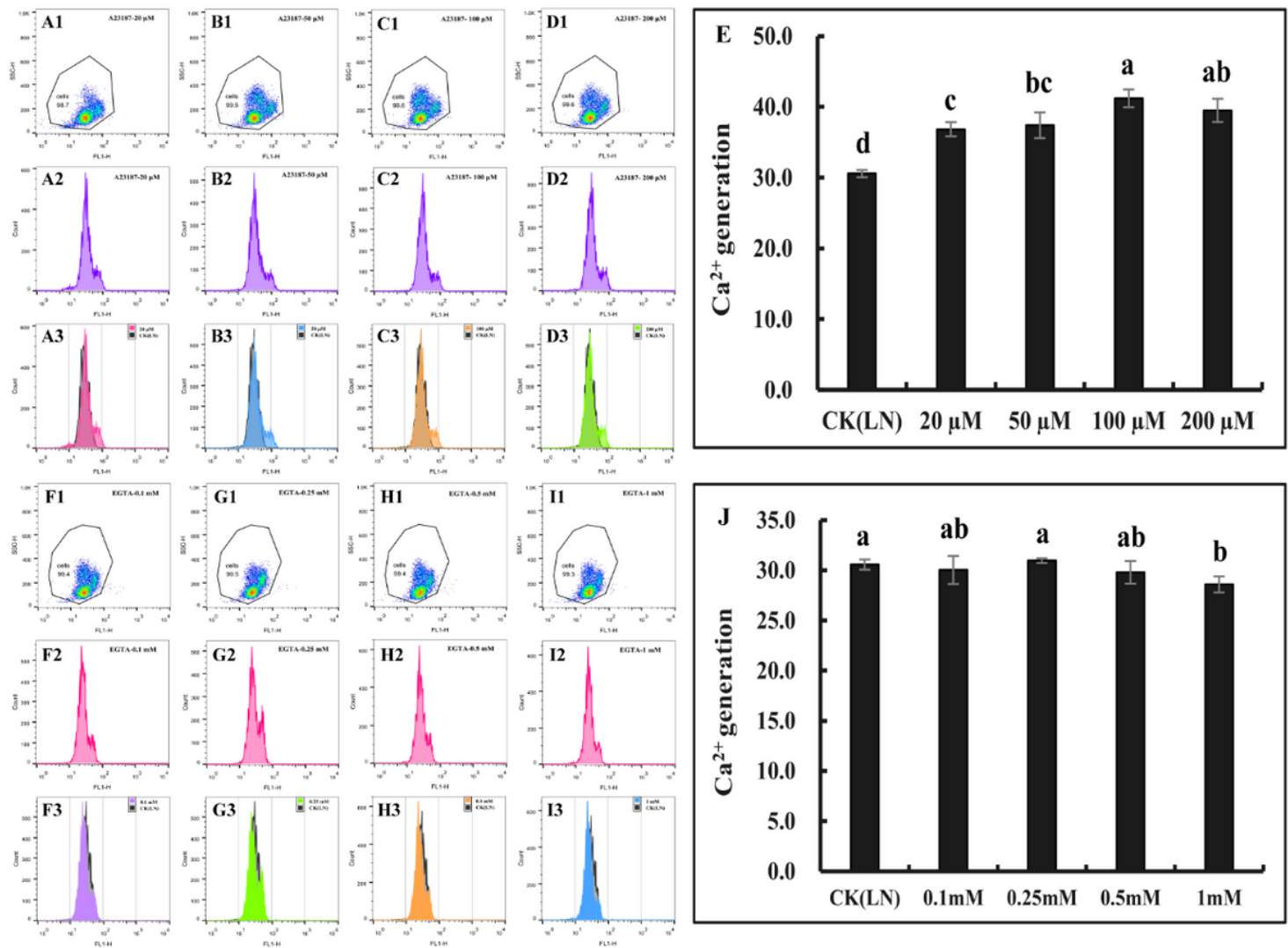
Figure 2

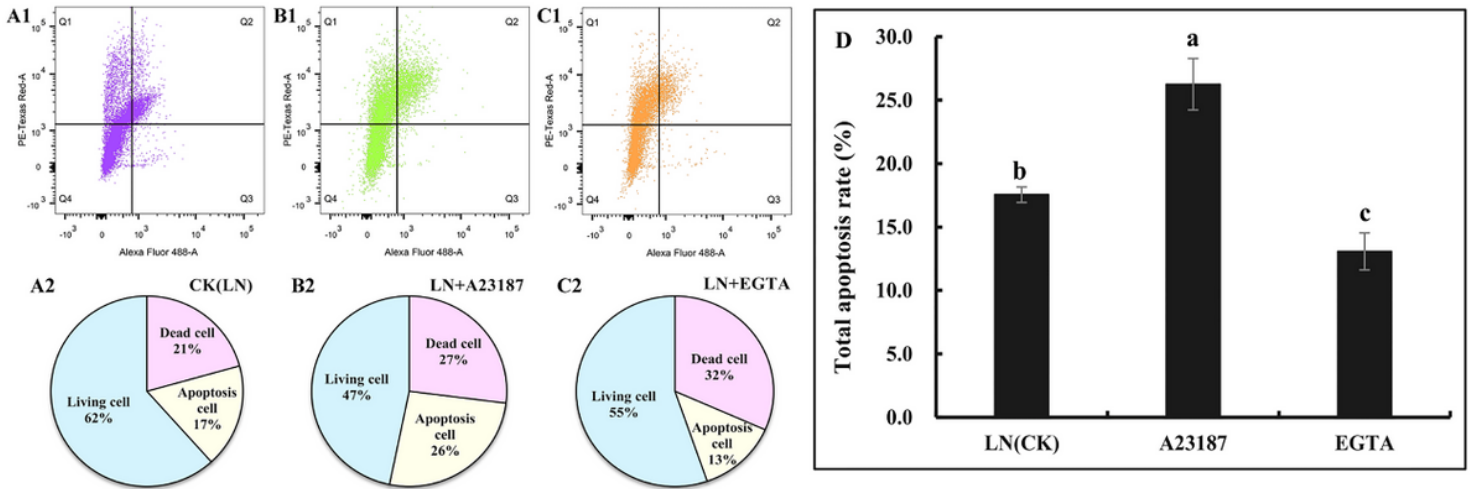**Changes of endogenesis Ca^{2+} content in pollen before and after exogenous Ca^{2+} regulator treatments.**

Notes: Different letters indicate significant differences among different treatment at the 0.05 level (P< 0.05, Duncan's multiple range test). A-20μM A23187; B-50μM A23187; C-100μM A23187; D-200μM A23187; E- the changes of endogenesis Ca^{2+} content before and after A23187 treatment; F-0.1mM EGTA; G-0.25 mM EGTA; H-0.5 mM EGTA; I-1mM EGTA; J- the changes of endogenesis Ca^{2+} content before and after EGTA treatment; 1- the flow cytometry maps; 2- the flow spectrogram of Fluo-3 AM fluorescence (Ca^{2+} fluorescent probe); 3- the comparison spectrograms. CK (LN): pollen thawed at room temperature after cryopreservation.
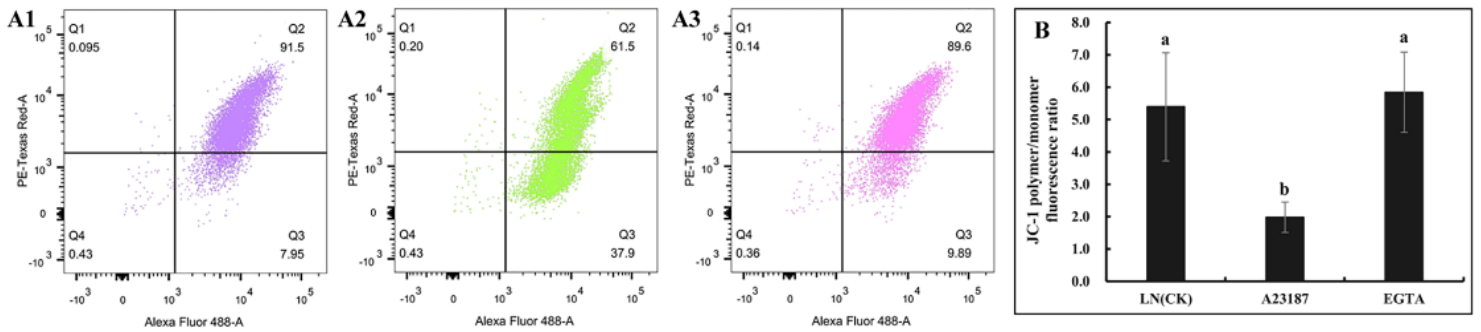
Figure 3

Changes of mitochondrial membrane potential in pollen before and after exogenous Ca^{2+} regulator treatments.
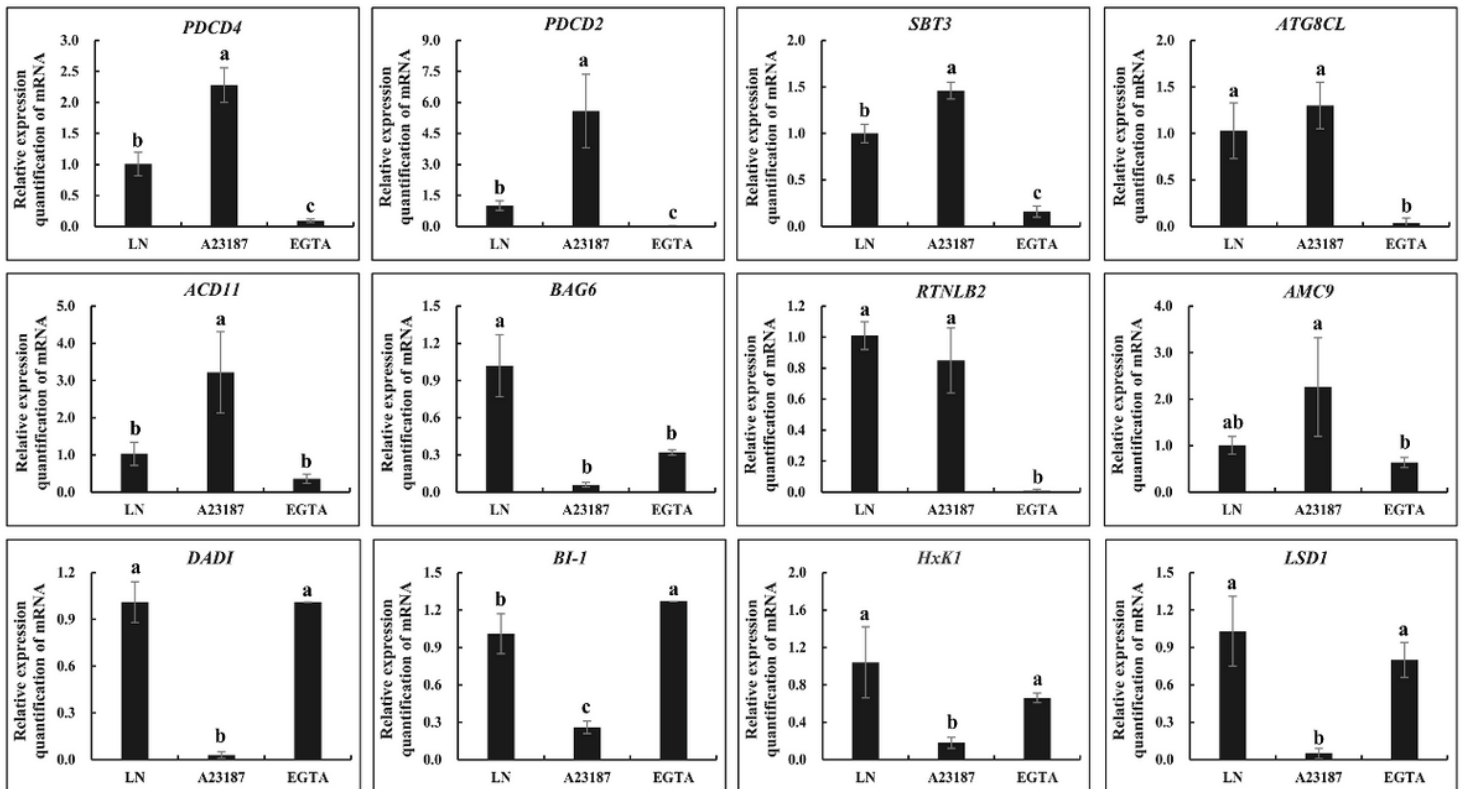
Notes: Different letters indicate significant differences among different treatment at the 0.05 level (P< 0.05, Duncan's multiple range test). A1- LN preserved pollen without running water thaw treatment (CK); A2-LN+A23187; A3-LN+EGTA; B- the changes of JC-1 monomer and polymer ratio. CK (LN): pollen thawed at room temperature after cryopreservation; A23187: pollen supplemented with A23187 after cryopreservation; EGTA: pollen supplemented with EGTA after cryopreservation.

Figure 4

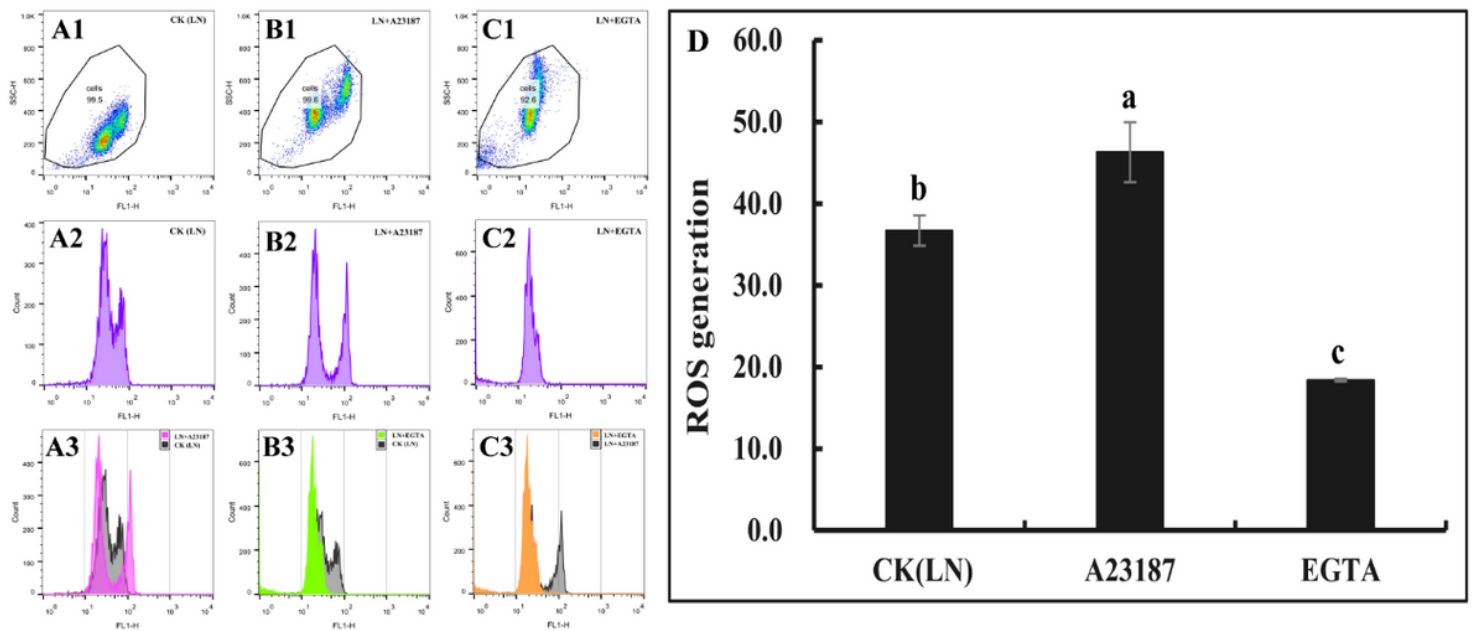Changes of caspase-like protease in pollen before and after exogenous Ca^{2+} regulator treatments.

Notes: Different letters indicate significant differences among different treatment at the 0.05 level (P< 0.05, Duncan's multiple range test). A- caspase-3-like; B- caspase-9-like. CK (LN): pollen thawed at room temperature after cryopreservation; A23187: pollen supplemented with A23187 after cryopreservation; EGTA: pollen supplemented with EGTA after cryopreservation.

Figure 5

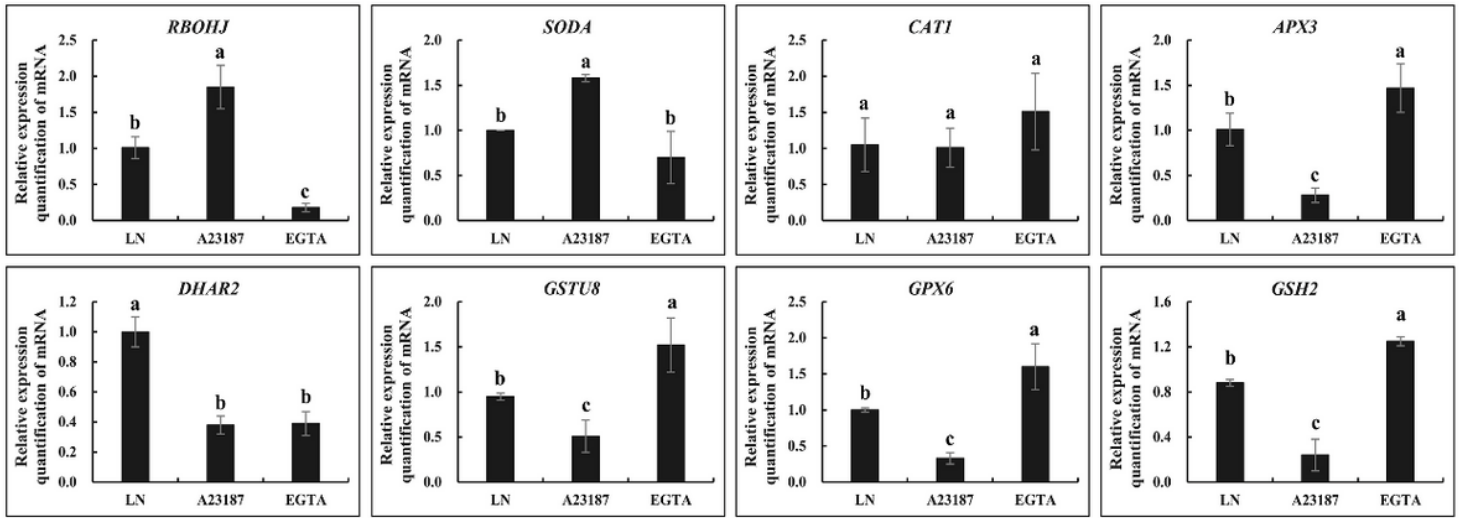**Changes of apoptosis rate in pollen before and after exogenous Ca^{2+} regulator treatments.**

Notes: Different letters indicate significant differences among different treatment at the 0.05 level (P< 0.05, Duncan's multiple range test). A-LN preserved pollen without running water thaw treatment (CK); B-LN+A23187; C-LN+EGTA; D- the changes of apoptosis rate; 1- the flow diagrams of Annexin V-FITC and PI double staining; 2- the proportions of dead cells, living cells, and apoptotic cells of pollen. CK (LN): pollen thawed at room temperature after cryopreservation; A23187: pollen supplemented with A23187 after cryopreservation; EGTA: pollen supplemented with EGTA after cryopreservation.

Figure 6

Changes of PCD gene in pollen with and without Ca^{2+} regulator after cryopreservation.

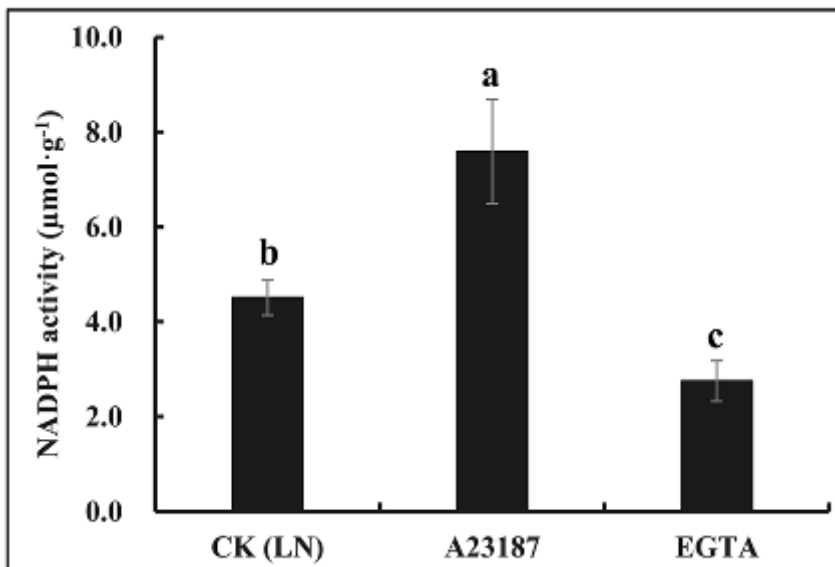Notes: Different letters indicate significant differences among different treatment at the 0.05 level (P< 0.05, Duncan's multiple range test). LN: pollen thawed at room temperature after cryopreservation; A23187: pollen supplemented with A23187 after cryopreservation; EGTA: pollen supplemented with EGTA after cryopreservation.

Figure 7

Changes of endogenesis ROS content in pollen before and after exogenous Ca^{2+} regulator treatments.

Notes: Different letters indicate significant differences among different treatment at the 0.05 level (P< 0.05, Duncan's multiple range test). A- CK (LN); B- LN+A23187; C-LN+EGTA; D −ROS content; 1- the flow cytometry maps; 2- the flow spectrogram of DCFH-DA fluorescence (ROS fluorescent probe); 3- the comparison spectrograms. CK (LN): pollen thawed at room temperature after cryopreservation; A23187: pollen supplemented with A23187 after cryopreservation; EGTA: pollen supplemented with EGTA after cryopreservation.

Figure 8

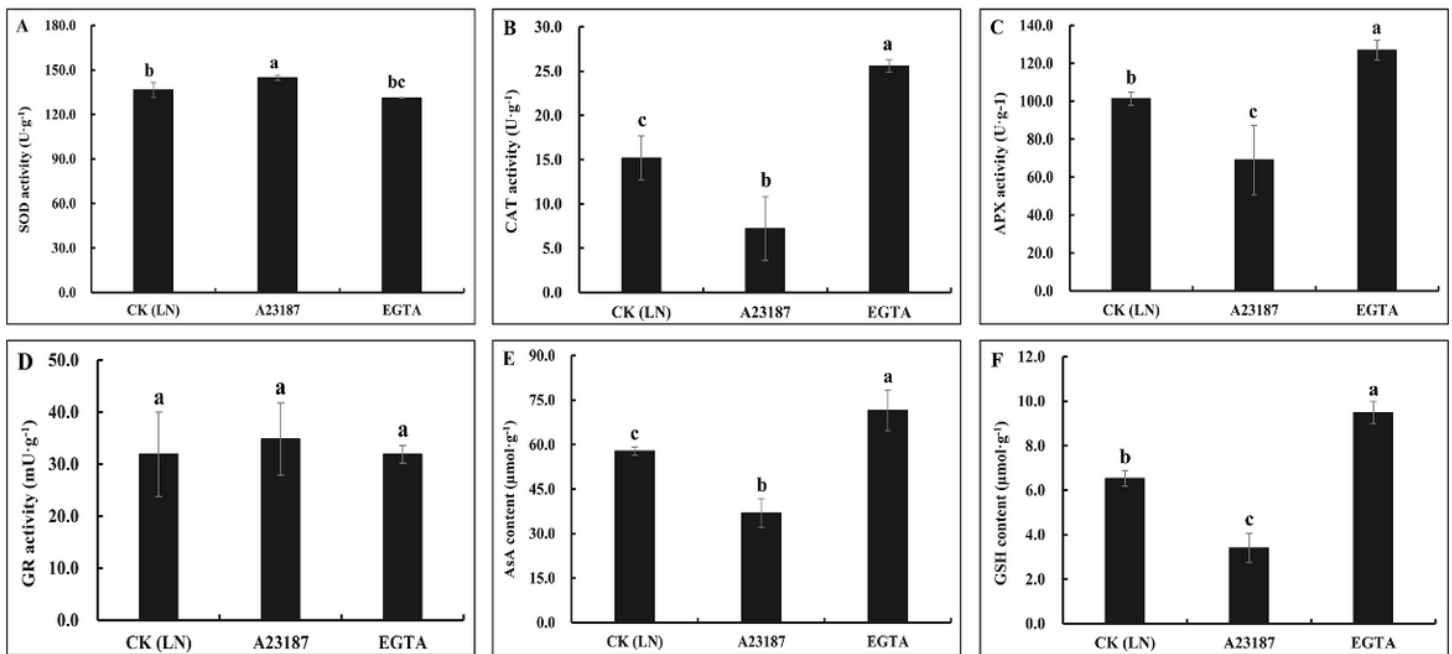Changes of ROS related genes in pollen with and without Ca^{2+} regulator after cryopreservation.

Notes: Different letters indicate significant differences among different treatment at the 0.05 level (P< 0.05, Duncan's multiple range test). LN: pollen thawed at room temperature after cryopreservation; A23187: pollen supplemented with A23187 after cryopreservation; EGTA: pollen supplemented with EGTA after cryopreservation.

Figure 9

Changes of ROS production enzyme (NADPH oxidase) in pollen before and after exogenous Ca^{2+} regulator treatments.

Notes: Different letters indicate significant differences among different treatment at the 0.05 level (P< 0.05, Duncan's multiple range test). CK (LN): pollen thawed at room temperature after cryopreservation; A23187: pollen supplemented with A23187 after cryopreservation; EGTA: pollen supplemented with EGTA after cryopreservation.
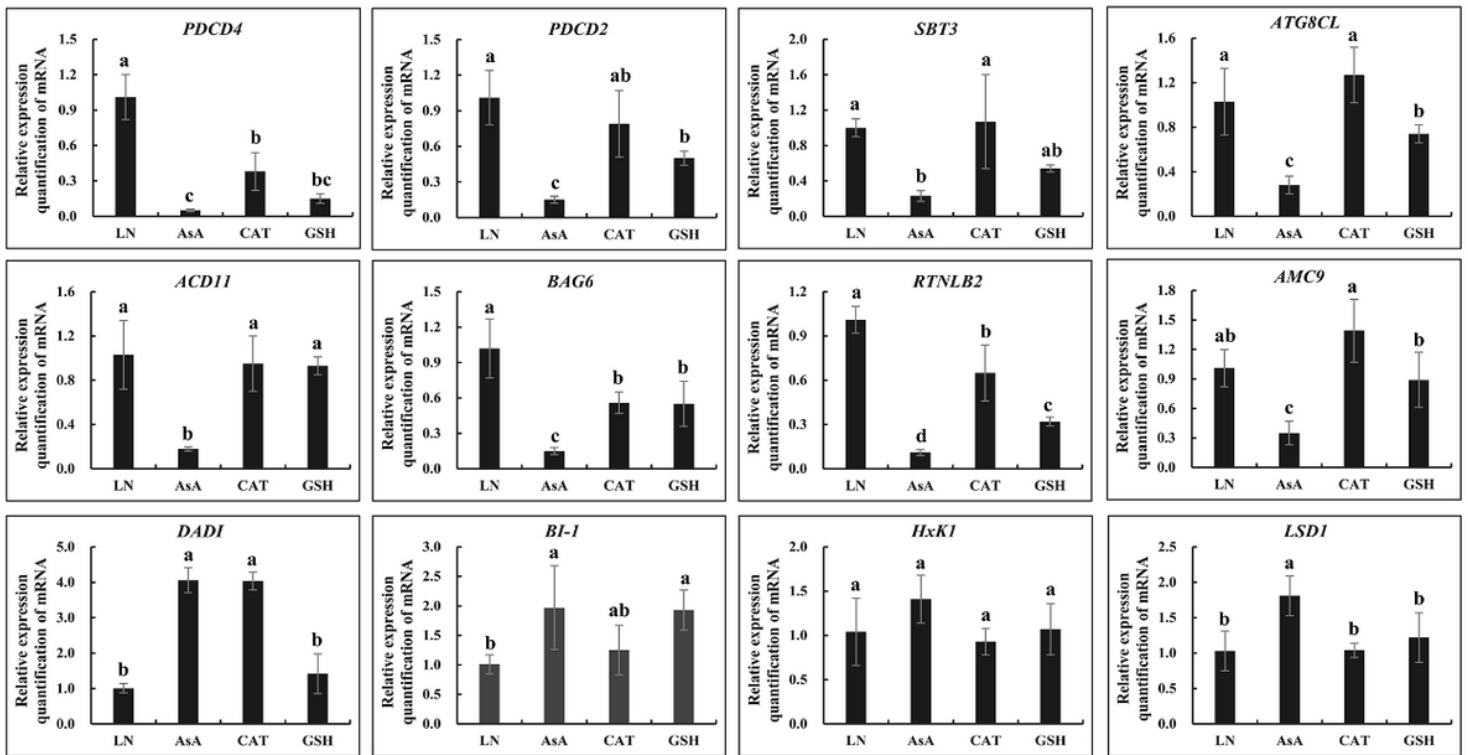

Figure 10

Changes of ROS clearance index in pollen before and after exogenous Ca^{2+} regulator treatments.

Notes: Different letters indicate significant differences among different treatment at the 0.05 level (P< 0.05, Duncan's multiple range test). A-SOD; B-CAT; C-APX; D-GR; E-AsA; F-GSH. CK (LN): pollen thawed at room temperature after cryopreservation; A23187: pollen supplemented with A23187 after cryopreservation; EGTA: pollen supplemented with EGTA after cryopreservation.

Figure 11

Changes of PCD gene in pollen with and without ROS regulator after cryopreservation.

Notes: Different letters indicate significant differences among different treatment at the 0.05 level (P< 0.05, Duncan's multiple range test). LN: pollen thawed at room temperature after cryopreservation; AsA: pollen supplemented with AsA after cryopreservation; CAT: pollen supplemented with CAT after cryopreservation; GSH: pollen supplemented with GSH after cryopreservation.

Supplementary Files

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