

## Heat Tolerance of Mansailong Pomelo Seeds at Different Developmental Stages (Postprint)

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**Date:** 2023-12-24T00:00:00+00:00

### Abstract

Global warming has led to frequent occurrences of extreme high temperatures, inevitably exposing plant seeds to high temperature stress environments. To investigate the physiological basis of seed heat tolerance, this study utilized intermediate-type seeds of ‘Mansailong’ pomelo (*Citrus maxima* ‘Mansailong’) as experimental material, subjected seeds at different developmental stages to high temperature treatment, and simultaneously monitored morphological changes, soluble protein and heat-stable protein contents, and alterations in cellular ultrastructure across various developmental periods. The results demonstrated that: (1) Throughout the entire developmental period from 23 to 49 weeks after anthesis, seed water content decreased significantly, fresh weight increased markedly, and the percentage of dry weight to fresh weight also exhibited obvious enhancement; these indices all underwent rapid changes around 31 weeks after anthesis and stabilized by 41 weeks after anthesis. (2) Seeds acquired complete seedling establishment capacity and preliminary heat tolerance at 29 weeks after anthesis, after which heat tolerance increased progressively and improved rapidly between 37 and 49 weeks after anthesis. Parallel to the changes in seed heat tolerance, soluble protein and heat-stable protein contents in seeds displayed a continuous increasing trend from 23 to 49 weeks after anthesis, and correlation analysis revealed that the accumulation of both was significantly positively correlated with seed heat tolerance throughout the developmental process. (3) Ultrastructural observations discovered that as seeds developed, embryonic axis cell volume gradually decreased, lipid bodies in cells progressively increased and became more regularly arranged, mitochondria gradually diminished, while vacuoles enlarged from small to large and later-stage vacuoles were filled with black flocculent material. These results indicate that ‘Mansailong’ pomelo seeds achieved physiological maturity at 41 weeks after anthesis, without an obvious maturation drying process; their heat tolerance was acquired and progressively enhanced during development until the late seed development stage; the increase in soluble protein and heat-stable protein con-

tents and changes in cellular ultrastructure made significant contributions to the development of seed heat tolerance.

## Full Text

## Preamble

DOI: 10.11931/guihaia.gxzw202308024

## Study on High Temperature Tolerance of *Citrus maxima* ‘Mansailong’ Seeds at Different Developmental Stages

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## Abstract

Global warming has led to increasingly frequent extreme high temperatures, inevitably exposing plant seeds to high temperature stress. To investigate the physiological basis of seed thermotolerance, we used intermediate-type seeds of *Citrus maxima* ‘Mansailong’ as experimental material, subjected seeds at different developmental stages to high temperature treatment, and simultaneously examined morphological changes, soluble and heat-stable protein contents, and ultrastructural changes. The results showed: (1) Throughout development from 23 to 49 weeks after flowering (WAF), seed moisture content decreased significantly while fresh weight increased markedly, and the dry weight/fresh weight percentage also increased substantially. These parameters changed rapidly around 31 WAF and stabilized by 41 WAF. (2) Seeds acquired full seedling formation capacity and preliminary high temperature tolerance at 29 WAF, after which thermotolerance increased gradually and improved rapidly between 37–49 WAF. Similar to the changes in thermotolerance, soluble and heat-stable protein contents showed continuous increasing trends from 23–49 WAF. Correlation analysis revealed that accumulation of these proteins was significantly positively correlated with seed thermotolerance throughout development. (3) Ultrastructural observations revealed that as seeds developed, embryonic axis cell volume gradually decreased, lipid bodies increased and became more regularly arranged, mitochondria decreased, and vacuoles enlarged while becoming filled with black flocculent material in later stages. These results indicate that *C. maxima* ‘Mansailong’ seeds reach physiological maturity at 41 WAF without an obvious maturation drying process. Their high temperature tolerance is acquired and gradually enhanced during development until late seed development stages. The increase in soluble and heat-stable protein contents and changes

in cellular ultrastructure make important contributions to the development of seed thermotolerance.

**Keywords:** high-temperature stress, intermediate seeds, cellular ultrastructure, soluble protein, heat-stable protein, seed development

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## Introduction

Seed development represents the initial stage of plant ontogeny and is extremely sensitive to environmental conditions. Seed quality not only affects the seeds themselves but may also influence the growth and development of the next generation (Mao et al., 2001). With global warming, high temperatures and extreme heat events are becoming more frequent, inevitably exposing plants to high temperature environments and subjecting seed development to heat stress. High temperatures during seed development profoundly affect seed quality and yield. For example, when rice seeds are exposed to high temperatures during development, their longevity, moisture content, and germination capacity are significantly affected (Rahman & Ellis, 2019). Gao (2015) compared rapeseed varieties with different thermotolerances and found that after high temperature treatment, germination potential and rate were significantly positively correlated with per-plant yield and total plant dry weight, while germination index and rate showed extremely significant positive correlations with yield and thousand-seed weight. These findings demonstrate that seed thermotolerance is related to plant growth status—better plant growth and higher yield correlate with stronger seed thermotolerance.

Currently, research on thermotolerance at different seed developmental stages remains scarce. However, seed thermotolerance is clearly closely related to desiccation tolerance, and insights from desiccation tolerance studies provide valuable guidance. Seed thermotolerance may be gradually acquired during development, with seeds at different stages exhibiting varying levels of tolerance. Moreover, seeds with different storage characteristics show distinct developmental patterns: orthodox seeds undergo maturation drying in late development, with reduced cellular metabolic activity, cellular dedifferentiation, and accumulation of heat-stable and soluble proteins (Prieto-Dapena et al., 2006), while dry matter accumulation ceases and moisture content drops sharply in the final developmental stage. In contrast, recalcitrant seeds show continuous dry matter accumulation throughout development, maintaining high moisture content even at shedding (Bewley et al., 2013). Soluble proteins are also important for seed germination, as increased content not only maintains lower osmotic potential to facilitate water uptake but also enhances adaptation to adverse conditions (Qin et al., 2022). Soluble proteins protect biomembranes and macromolecules under stress conditions and are critical for acquiring stress resistance (Wehmeyer et al., 1996; Török et al., 2001), with heat-stable proteins being particularly closely associated with thermotolerance (Li et al., 2010). Studies on thermotol-

erance in orthodox bean seeds found that appropriate salicylic acid treatment increased soluble protein content and enhanced thermotolerance (Qin et al., 2022). Conversely, recalcitrant seeds native to tropical rainforests may lack or insufficiently accumulate heat-stable proteins in late development (Farrant et al., 1992), showing obvious deficiency in stress resistance. Moreover, as germination initiates, heat-stable protein content gradually decreases and thermotolerance declines (Burke & O'Mahony, 2001). Based on these observations, we hypothesize that acquisition of seed thermotolerance is associated with cellular structure, metabolic activity, and synthesis of protective proteins during seed development.

Pomelo is a renowned tropical/subtropical fruit with extensive cultivation, high yield, and good storability; China ranks first worldwide in both planting area and production. As the origin and distribution center of pomelo, China possesses rich germplasm resources and a long cultivation history (Shen et al., 1998). Previous studies have shown that *C. maxima* 'Mansailong' seeds are intermediate-type seeds (Wen et al., 2010). While their desiccation tolerance is relatively close to that of orthodox seeds, their thermotolerance is more similar to recalcitrant seeds, yet not identical, indicating certain unique characteristics (An et al., 2023). This study investigates changes in thermotolerance during seed development in *C. maxima* 'Mansailong', explores the physiological mechanisms underlying thermotolerance formation, and provides a scientific basis for developing anti-high temperature technologies.

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## Materials and Methods

### Experimental Material

The study used *Citrus maxima* 'Mansailong', widely cultivated in Xishuangbanna, which flowers from mid-January to late March annually with fruit maturation in mid-October (Yang, 2001). Research indicates that *C. maxima* 'Mansailong' seeds reach physiological maturity at 180 days after flowering, completing morphological establishment and acquiring germination capacity (Xue & Wen, 2015). We observed that in 2022, concentrated flowering at the Economic Plant Promotion Station Orchard of Xishuangbanna Tropical Botanical Garden occurred around January 6. Accordingly, from June to December 2022, we marked seed development by weeks after flowering (WAF), selecting fruits of similar size and color every two weeks. Ten to fifteen fruits were harvested, and after removing peel, pulp, and seed coat, approximately 1,300 seeds were obtained. Following collection of basic data including thousand-seed weight and moisture content, some seeds were used for thermotolerance evaluation while others were reserved for ultrastructural observation and protein analysis.

### 1.2.1 Determination of Basic Seed Parameters

**Fresh weight measurement:** One hundred seeds were randomly selected and weighed, with ten replicates averaged.

**Moisture content determination:** Following International Seed Testing Association procedures (ISTA, 1996), one seed was randomly selected with eight replicates, oven-dried at  $(103 \pm 2)^{\circ}\text{C}$  for  $(17 \pm 1)$  h. Moisture content was expressed on a fresh weight basis: Moisture content (%) = (fresh weight - dry weight) / fresh weight  $\times$  100.

**Seed viability assessment:** Seeds were sown in 1% agar Petri dishes with the germination end upward, with 25 seeds per dish constituting one replicate and six replicates per treatment. Germination was observed and recorded weekly; emergence of radicle approximately 0.5 cm was considered germination/survival, and formation of morphologically normal seedlings was considered successful seedling establishment. The germination period was approximately one month, with ungerminated seeds examined for rot at experiment termination. Final germination rate and seedling formation rate were used to measure seed viability.

### 1.2.2 Evaluation of Thermotolerance at Different Developmental Stages

Previous studies showed that *C. maxima* ‘Mansailong’ seeds achieved highest germination rates and fastest germination at 30°C (optimal temperature), while germination was severely inhibited at 40°C (An et al., 2023). Since ground surface temperatures in Xishuangbanna open areas frequently reach or exceed 40°C (Liu et al., 2000), we used continuous high temperature treatment at 40°C to evaluate thermotolerance. Seeds were sown in 1% agar Petri dishes and placed in a 40°C incubator for various durations. After high temperature treatment, dishes were transferred to a 30°C incubator for cultivation, with germination and seedling formation monitored as described above. Maximum high temperature treatment duration ranged from 12 h to 1,032 h depending on developmental stage. Thermotolerance at different developmental stages was evaluated based on changes in survival and seedling formation rates after high temperature treatment.

### 1.2.3 Determination of Soluble and Heat-Stable Proteins

**Extraction:** From seeds at different developmental stages, 10–20 seeds were selected, seed coats removed with forceps, chopped, placed in cryovials, and stored at -80°C. Before analysis, approximately 0.1 g fresh weight of sample was ground in a mortar with 0.5 mL PBS buffer on ice, transferred to a 1.5 mL centrifuge tube, then rinsed with another 0.5 mL PBS buffer. After thorough mixing, samples were centrifuged twice at 15,000 g for 15 min at 4°C. Approximately 400  $\mu$ L of supernatant was collected, with half used for soluble protein determination and the other half heated at 95°C for 10 min in a water bath.

After cooling, samples were centrifuged at 15,000 g for 15 min at 4°C, with the supernatant collected for heat-stable protein analysis (Thierry et al., 1999).

**Standard curve preparation:** Protein content was determined using Bradford's (1976) Coomassie brilliant blue method with a microplate reader (Chen et al., 2018). Fifty mg of bovine serum albumin was dissolved in PBS and diluted to 50 mL to prepare a  $1 \text{ mg} \cdot \text{mL}^{-1}$  standard protein solution, which was further diluted with PBS to concentrations of 0, 100, 200, 400, 600, 800, and  $1,000 \text{ g} \cdot \text{mL}^{-1}$ . After adding Coomassie brilliant blue G-250 stain, absorbance was measured at 595 nm to establish a standard curve.

**Measurement:** For soluble protein, 25  $\mu\text{L}$  sample was diluted with 75  $\mu\text{L}$  extraction buffer, then 0.5 mL Coomassie brilliant blue stain was added. After mixing and standing for 5–20 min, absorbance was measured at 595 nm with a microplate reader and protein content calculated from the standard curve. For heat-stable protein, 50  $\mu\text{L}$  sample was diluted with equal extraction buffer, 0.5 mL stain added, mixed, stood for 5–20 min, and OD measured at 595 nm for protein content calculation.

#### 1.2.4 Ultrastructural Observation of Embryonic Axis Cells at Different Developmental Stages

The primary function of seeds is germination and seedling development, with the embryonic axis being the most critical component for successful seedling establishment. Damage to the embryonic axis under stress most easily leads to viability loss (Cheng et al., 1991; Priestley, 1986). Previous ultrastructural studies of seeds have often focused on the embryo (for small-seeded species) or embryonic axis (for large-seeded species), such as in maize (Wen et al., 2009) and *Livistona chinensis* (Wen, 2011). Accordingly, five seeds from each developmental stage were sampled, seed coats removed with a scalpel and forceps, and the tip containing the embryonic axis excised. Samples were fixed in 3 mL electron microscopy fixative and stored at 4°C. For observation, approximately 1 mm radicle tissue was excised, fixed with 3.5% glutaraldehyde and 1% osmium tetroxide, dehydrated with graded acetone, and embedded in epoxy resin for one week. After processing, semi-thin sections were cut and screened under a light microscope before preparing ultrathin sections (Wang et al., 2014). Ultrathin sections were stained with lead citrate and uranyl acetate before electron microscopy examination to investigate how cellular morphology, structure, and organelle development affect seed thermotolerance.

#### 1.2.5 Statistical Analysis

Data were processed using Microsoft Excel 2010 to calculate means and standard errors, with graphs prepared using Origin 2023. One-way ANOVA was performed using SPSS 22.0 at  $P < 0.05$ . Probit analysis was used to calculate the high temperature treatment duration causing 50% mortality to evaluate seed thermotolerance.

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## Results

### 2.1 Changes in Fresh Weight, Dry/Fresh Weight Ratio, and Moisture Content During Seed Development

Significant changes and stage-specific characteristics were observed in fresh weight, dry/fresh weight ratio (DW/FW), and moisture content of *C. maxima* ‘Mansailong’ seeds from 23–49 WAF [Figure 1: see original paper]. Fresh weight increased significantly from 23–35 WAF, then decreased from 37 WAF and remained relatively stable. Moisture content remained high (approximately 82%) at 23, 25, and 27 WAF, then decreased significantly from 29–39 WAF ( $P < 0.05$ ) at an average rate of 2.8 percentage points per week, stabilizing from 41 WAF at approximately 42%. The DW/FW ratio showed an overall pattern of increase followed by stabilization, remaining around 17% at 23–27 WAF, then gradually increasing to 57% by 41 WAF (an increase of nearly 39 percentage points) before stabilizing. These results indicate that *C. maxima* ‘Mansailong’ seeds reach physiological maturity around 41 WAF, with moisture content stabilizing at approximately 42%. The persistently high moisture content at maturity suggests these seeds lack a maturation drying process.

*All data are expressed as means  $\pm$  SE of 10 or 8 replicates.*

**Figure 1.** Changes in fresh weight, DW/FW ratio, and moisture content of seeds at different developmental stages

### 2.2 Changes in Thermotolerance During Seed Development

Seeds at different developmental stages showed extreme differences in tolerance to continuous 40°C high temperature. Early developmental stages exhibited very poor thermotolerance; for example, at 23 WAF seeds had germination capacity (43% germination rate) but could not form seedlings, even without high temperature treatment. Seedling formation capacity was not acquired until 27 WAF, when 15% of seeds could develop into seedlings under optimal temperature (30°C), but this decreased to less than 4% after 12 h high temperature treatment, with all seeds dying after 24 h treatment. At 29 WAF, seedling formation rate reached 90%, and seeds only lost viability completely after 90 h high temperature treatment, indicating acquisition of preliminary thermotolerance. Thereafter, thermotolerance increased gradually with maturation; seeds at 49 WAF still showed 84% seedling formation rate after 600 h high temperature treatment [Figure 2: see original paper].

*All data are expressed as means  $\pm$  SE of 6 replicates of 25 seeds. WAF indicates weeks after flowering, the same below.*

**Figure 2.** Changes in high-temperature tolerance during *Citrus maxima* ‘Mansailong’ seed development

Since seed survival and seedling formation rates exceeded 50% at 25 and 29 WAF respectively after high temperature treatment, we used probit regression analysis based on survival and seedling formation rates to calculate the median lethal time ( $LT_{50}$ ) for continuous 40°C treatment in subsequent developmental stages. The  $LT_{50}$  gradually increased with seed development. From 25–31 WAF,  $LT_{50}$  increased from 8 h to 111 h (average increase of 18 h per week). From 31–37 WAF, the increase was slower (average 6 h per week). The fastest improvement occurred from 39–41 WAF, with  $LT_{50}$  increasing dramatically by 296 h in just two weeks. Thermotolerance continued to increase thereafter, reaching nearly 800 h in the final stage. Before 39 WAF,  $LT_{50}$  values calculated from survival and seedling formation rates showed almost no difference; after 39 WAF, only minor differences appeared [Figure 3: see original paper].

**Figure 3.** Changes in median lethal time ( $LT_{50}$ ) of seeds at different developmental stages under high temperature treatment at 40°C (Means  $\pm$  SE)

### 2.3 Changes in Soluble and Heat-Stable Protein Contents During Seed Development

Because moisture content varied greatly among developmental stages, protein contents were calculated on a dry weight basis. Both soluble and heat-stable protein contents increased during seed development. The period from 25–31 WAF was a rapid accumulation phase for soluble protein, increasing from 7.21  $\text{mg} \cdot \text{g}^{-1}$  to 38.35  $\text{mg} \cdot \text{g}^{-1}$  (average increase of 5.19  $\text{mg} \cdot \text{g}^{-1}$  per week,  $P < 0.05$ ). Accumulation slowed thereafter to only 0.67  $\text{mg} \cdot \text{g}^{-1}$  per week, with no significant differences between adjacent stages. Heat-stable protein showed a similar trend, with 25–31 WAF also being a rapid accumulation period, increasing by 18.77  $\text{mg} \cdot \text{g}^{-1}$  ( $P < 0.05$ ). No significant changes occurred from 35–45 WAF, then increased by 4.62  $\text{mg} \cdot \text{g}^{-1}$  at 47 WAF ( $P < 0.05$ ) before stabilizing [Figure 4: see original paper]. Thus, 25–31 WAF represents a critical stage for rapid accumulation of both soluble and heat-stable proteins. Comparison with thermotolerance changes shows that this stage coincides with rapid increases in seed thermotolerance, indicating that accumulation of these proteins plays an important role in acquiring thermotolerance.

**Figure 4.** Changes in content of soluble protein and heat-stable protein during seed development (Means  $\pm$  SE)

### 2.4 Ultrastructural Changes in Embryonic Axis Cells at Different Developmental Stages

Seeds at different developmental stages exhibited distinct cellular structural characteristics. In early development (23 WAF), embryonic axis cells were large with large nuclei, clear nuclear membranes and nucleoli, small and few lipid bodies distributed irregularly in the cytoplasm, numerous mitochondria, and numerous scattered small vacuoles, indicating young cells with vigorous metabolism [FIGURE:5: A-C]. By mid-development (37 WAF), obvious changes occurred:

lipid bodies enlarged and aligned along the inner cell wall, vacuoles gradually enlarged with small amounts of black flocculent material, and numerous mitochondria indicated active metabolism and storage material accumulation [FIGURE:5: D-F]. In late development (47 WAF), embryonic axis cells were smaller with few observable mitochondria; black flocculent material in vacuoles increased substantially, almost filling entire vacuoles; cells showed high maturity with numerous lipid bodies accumulated and regularly arranged along the plasma membrane [FIGURE:5: G-I]. These observations indicate that *C. maxima* ‘Mansailong’ seed development involves metabolic shutdown and storage material accumulation processes that contribute importantly to thermotolerance acquisition.

A-C. 23 WAF; D-F. 37 WAF; G-I. 47 WAF. CW. Cell wall; N. Nucleus; NM. Nuclear membrane; M. Mitochondria; LB. Lipid body; G. Golgi body; V. Vacuole.

**Figure 5.** Ultrastructure of embryonic axis cells in seeds at different developmental stages

## 2.5 Correlation Analysis of Developmental Indicators in *C. maxima* ‘Mansailong’ Seeds

During seed development,  $LT_{50}$  values calculated from survival and seedling formation rates showed significant positive correlations with soluble protein, heat-stable protein, and DW/FW ratio, and negative correlations with fresh weight and moisture content. Moisture content showed extremely significant negative correlations with DW/FW ratio, heat-stable protein, and soluble protein. DW/FW ratio showed extremely significant positive correlations with both heat-stable and soluble proteins, which also showed extremely significant positive correlation with each other .

**Table 1.** Correlation analysis of various indicators during the development of *Citrus maxima* ‘Mansailong’ seeds

Index	Survival	Seedling	Fresh weight	Moisture content	DW/FW	Heat-stable protein	Soluble protein
Survival	1.000**	-	-0.724**	-0.695*	0.724**	0.695*	-1.000**
Seedling	0.697*	1.000**	0.811**	-0.986**	0.986**	-	-
Fresh weight	0.738**	0.813**	1.000**	-0.978**	0.978**	0.995**	-
Moisture content	-	-	-	1.000**	-	-	-
DW/FW	-	-	-	-	1.000**	-	-

Index	Survival	Seedling weight	Fresh weight	Moisture content	DW/FW	Heat-stable protein	Soluble protein
Heat-stable protein	-	-	-	-	-	-	-
Soluble protein	-	-	-	-	-	-	-

*Note:* indicates significant differences ( $P < 0.05$ ); \*\* indicates extremely significant differences ( $P < 0.01$ ).\*

## Discussion and Conclusion

Seed maturation includes both physiological and morphological aspects. Physiological maturation is characterized by stable dry matter content, reduced moisture content, and acquired germination capacity, while morphological maturation involves stabilization of shape, size, and color (Qi, 2009). Hamilton et al. (2007) reported that *Citrus garrawayi* seeds reached maturity when moisture content decreased from  $(82 \pm 1) \pm 1\%$ . Our results for *C. maxima* 'Mansailong' are consistent, with moisture content decreasing from approximately 82% at 23 WAF to 42% at 41 WAF, after which no significant changes occurred. Additionally, fresh weight and DW/FW ratio stabilized at 41 WAF, further indicating physiological maturity. However, fresh weight became somewhat unstable after 35 WAF, even decreasing at 47 and 49 WAF, likely because few fruits remained available in the orchard for sampling, and harvested fruits were smaller.

Bewley et al. (2013) demonstrated that orthodox seeds undergo a maturation drying stage before shedding, when dry matter accumulation ceases and moisture content and fresh weight decrease significantly. Recalcitrant seed development differs, with continuous dry matter accumulation until shedding and persistently high moisture content without obvious maturation drying. Our results show that *C. maxima* 'Mansailong' differs from both orthodox and recalcitrant seeds in moisture content changes and dry matter accumulation: moisture content remained high (approximately 81%) in early development, then decreased gradually to a relatively stable level after 41 WAF, similar to recalcitrant seeds. Dry weight increased throughout development, while fresh weight first increased then decreased to a stable level, similar to orthodox seeds. Therefore, the developmental pattern of *C. maxima* 'Mansailong' seeds is intermediate between orthodox and recalcitrant seeds.

Seed desiccation tolerance is a quantitative trait. In orthodox seeds, desiccation tolerance develops gradually during seed development and may continue

strengthening after physiological maturity (Sun et al., 1993; Wen, 2008; Yang et al., 2002), while in recalcitrant seeds it peaks at physiological maturity then may decline slightly (Peng & Fu, 1994). Thermotolerance acquisition in *C. maxima* ‘Mansailong’ resembles orthodox seeds, gradually increasing from 25–41 WAF and rising sharply at 41 WAF (physiological maturity), after which thermotolerance continued increasing—similar to the desiccation tolerance development pattern in intermediate-type grapefruit seeds (Zhang, 2014). The enhancement of thermotolerance was almost synchronized with increases in soluble and heat-stable protein contents, as both showed continuous increasing trends from 23–49 WAF. Correlation analysis further demonstrated significant positive correlations between accumulation of these proteins and thermotolerance, indicating that increased soluble and heat-stable protein contents reduce high temperature damage to seed membranes and tissues, thereby enhancing thermotolerance. These findings align with previous research on the protective functions of these proteins (Fu, 2019; Li et al., 2010; Qin et al., 2022).

In early development of orthodox seeds, embryonic cells are large with small nuclei, uneven size, loose arrangement, large intercellular spaces, large and numerous vacuoles, and abundant organelles such as endoplasmic reticulum and mitochondria. In late development, cells become tightly and regularly arranged with small size and large nuclei, smaller or absent vacuoles, and accumulated protein and lipid bodies with reduced organelles (Wen et al., 2009). In recalcitrant seeds, early embryonic cells are loosely arranged, large with small nuclei, containing a large vacuole occupying most of the cell space, with abundant mitochondria and ribosomes. Mid-development cells are more tightly arranged, small with large nuclei, with large vacuoles transitioning to several small vacuoles, fewer organelles, and appearing lipid bodies. Late-stage peripheral cells are mature, occupied mostly by a vacuole filled with black granular material, with central positions composed of both young and mature cells (Wen, 2011). Our ultrastructural observations of *C. maxima* ‘Mansailong’ embryonic axis cells revealed that early and mid-development of intermediate seeds resembles orthodox seeds: early embryonic axis cells are large with small, few vacuoles and lipid bodies, numerous mitochondria, and vigorous metabolism; mid-development cells are smaller than early-stage cells, with more lipid bodies arranged along the inner cell membrane and black material appearing in vacuoles. Literature review indicates that such vacuoles rich in black material represent developing protein bodies (Klein & Pollock, 1968).

Late development of intermediate seeds differs from both orthodox and recalcitrant seeds but is more similar to recalcitrant seeds. In late development of *C. maxima* ‘Mansailong’, embryonic axis cells are smaller, with increased lipid bodies occupying most of the cell space; black material in vacuoles increased substantially but never formed true protein bodies at maturity, similar to mature cells of recalcitrant seeds in late development but different from orthodox seeds (Wen et al., 2009). This resembles findings from Wang (2020) on intermediate-type *Magnolia zenii* endosperm cell development. Therefore, from a developmental perspective, intermediate seeds are also intermediate between

recalcitrant and orthodox seeds.

Thermotolerance in *C. maxima* ‘Mansailong’ seeds is acquired gradually during development and correlates with changes in soluble and heat-stable protein contents and cellular ultrastructure. Early developmental stages show poor thermotolerance, with young, metabolically active cells containing few lipid bodies. Mid-development seeds have higher thermotolerance, with accumulating lipid bodies arranged along the inner cell membrane and black material appearing in vacuoles. Late-development seeds show maximum thermotolerance, with abundant lipid bodies occupying most of the cell space and vacuoles almost completely filled with black material. Thus, the process of seed development and maturation in *C. maxima* ‘Mansailong’ is also a process of acquiring and enhancing thermotolerance, with concurrent increases in lipid bodies and black vacuolar material playing important roles. We conclude that thermotolerance acquisition is inseparable from cellular morphological changes, metabolic changes, and storage material accumulation, representing the physiological basis of thermotolerance.

Based on storage characteristics, seeds can be classified as orthodox, recalcitrant, or intermediate. Among existing species, orthodox seeds predominate. In Dickie and Pritchard’s (2002) survey of 7,146 species, the vast majority (approximately 90%) were orthodox, approximately 7% were recalcitrant, and only 2% were intermediate. Liu et al. (2019) compiled data from the Royal Botanic Gardens, Kew’s Millennium Seed Bank, which included 24,781 plant species: 620 recalcitrant species (2.5%) and 149 intermediate species (0.6%). Intermediate seeds are relatively rare in nature, and the concept was proposed relatively recently, so research on this seed type remains insufficient and understanding is limited. Coffee and citrus are the two groups producing the most intermediate seeds and are currently the most studied. Since coffee originates from Africa, domestic research has focused more on citrus, particularly pomelo seeds. Previous work has concentrated on desiccation tolerance, low temperature tolerance, and storage tolerance, primarily for storage applications. Our focus on thermotolerance connects more with seed ecology, such as seed aging, longevity, survival, and germination in the wild. For pomelo seeds, our previous research has investigated cryopreservation (Zhang et al., 2014; Yan et al., 2014; Yang et al., 2021), desiccation tolerance development (Xue & Wen, 2015; Xue & Wen, 2018), cryotolerance development (Wen, 2018), cryotolerance among different varieties (Wen et al., 2010), and thermotolerance (An et al., 2023). This study examines thermotolerance development, detecting changes in thermotolerance during seed development and exploring connections with ultrastructural changes and accumulation of soluble and heat-stable proteins. These findings enhance our understanding of the physiological basis of seed thermotolerance and deepen knowledge of intermediate seeds.

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