



Physiological and molecular characteristics associated with the anti-senescence in *Camellia oleifera* Abel.

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Abstract

This study analyzed physiological and molecular characteristics associated with the resistance to aging or anti-senescence in *Camellia oleifera* Abel. Trees over 100 years old (ancient trees) were compared with those about 30 years old (mature trees). Total chlorophylls, chlorophyll *a/b* ratio, and hydrogen peroxide concentrations in ancient tree leaves were significantly higher than in their counterparts. Significantly higher activities of superoxide dismutase, peroxidase, and catalase were detected in ancient tree leaves. Nine Chl *a/b*-binding protein genes, 15 antioxidant enzyme genes, 21 hormone-related genes, and 301 stress-related genes were upregulated, and 42 protein-degradation genes were downregulated in ancient tree leaves. By increasing chlorophyll content and antioxidant enzyme activities and regulating the ageing-related genes expression, ancient *C. oleifera* leaves maintained remarkable vitality. Although further research is needed, our study may shed some light on how ancient *C. oleifera* trees can resist ageing and sustain their healthy growth.

Keywords: anti-ageing; anti-senescence; *Camellia oleifera*; plant senescence.

Introduction

Tree senescence is the natural decline observed in plants or specific organs during their growth and development, leading to the end of their life activities (Lim *et al.* 2007,

Chen and Dong 2016). Senescence occurs due to internal factors, such as hormones, transcription factors, aging-related genes, and metabolic alterations as well as a range of external factors, such as drought, high temperature, pathogen attack, pest infestation, and oxidative stress.

Highlights

- Ancient *Camellia oleifera* trees had higher chlorophyll contents and antioxidant enzyme activities
- Anti-senescence-related genes were mainly upregulated in ancient tree leaves
- Protein-degradation genes were largely downregulated in ancient tree leaves

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Abbreviations: ABA – abscisic acid; APX – ascorbate peroxidase; bHLH – basic helix-loop-helix; CAT – catalase; Chl – chlorophyll; Chl (*a+b*) – total chlorophyll content; CKX – cytokinin oxidase; CTK – cytokinin; ERF – ethylene-responsive transcription factor; HSD – honest significant difference test; HSP – heat-shock protein; MDA – malondialdehyde content; MYB – v-Myb avian myeloblastosis viral oncogene homolog; POD – peroxidase; ROS – reactive oxygen species; RT-qPCR – real-time quantitative PCR; SAUR – small auxin up RNA; SOD – superoxide dismutase.

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The action of some individual factors or in combination could lead to chlorophyll (Chl) degradation and hydrolysis of proteins, lipids, nucleic acids, starch, and other biological macromolecules (Balazadeh *et al.* 2010, Watanabe *et al.* 2013), resulting in the acceleration of the aging process, causing the death of an entire tree.

Currently, research on the causes and mechanism of tree senescence is ongoing. Several theories have been proposed including stomatal regulation theory, senescence gene regulation theory, active oxidation theory, nutrient deficiency theory, plant hormone regulation theory, and external stress and pest theory (Lee and Chen 2002). All these theories acknowledge that plant senescence and death are the universal laws of life development and an active and necessary process in plant morphogenesis, responses to environmental factors, and growth and development. These theories also indicate that tree senescence is formed under the combined action of many different factors. Meanwhile, due to different mechanisms and inducing factors of senescence (Chang *et al.* 2019), different plants have inconsistent senescence processes. When encountering several external stress and internal senescence-related factors, some plants could form corresponding defense mechanisms through antioxidant enzymes, transcription factors, and signal transduction pathways to prolong their senescence process. Such coordinated actions could be called anti-senescence or anti-aging strategies.

Camellia oleifera is a perennial small tree or shrub and a unique woody oil species (Chen *et al.* 2015a) distributed in low mountains and hills in Southern China across 14 provinces (Li *et al.* 2016). *C. oleifera* has a wide range of adaptations to poor fertility soils and drought environments, thus possessing exuberant vitality (Wang *et al.* 2015, Wu *et al.* 2020). It was common to find that more than 100 years old *C. oleifera* plants grow vigorously in natural forests. For example, in Hendong Country of Hunan Province, China (27°05'N, 112°56'E), some ancient *C. oleifera* trees over 120 years old, produced over 200 kg of fresh fruits per tree annually from 2014 to 2016. The question is what factors contribute to the remarkable vitality of these *C. oleifera* trees? Leaves are the primary organ for photosynthesis and are pivotal for plant growth and development. Leaf senescence was closely related to plant senescence (Lim *et al.* 2007). Regarding leaf aging, anatomy, ultrastructure, photosynthetic efficiencies,

chemical components, and the expression of aging-related genes were studied in *Platycladus*, *Ginkgo biloba*, and other tree species (Chang *et al.* 2019, Liu *et al.* 2019, Zhou *et al.* 2019, Turfan *et al.* 2020, Wang *et al.* 2020, Yan *et al.* 2021). However, research about *C. oleifera* trees in anti-senescence is scarce.

The objectives of this study were intended to investigate physiological and molecular characteristics associated with anti-senescence in *C. oleifera* through the comparison of two differently aged trees: more than 100 vs. about 30 years old and to provide some insight into how those over 100 years old trees can remain vigorous and maintain their growth and development with great vitality.

Materials and methods

Plant materials: *Camellia oleifera* trees belonging to two age groups: one was more than 100 years old called ancient trees, and the other was about 30 years old called mature trees (Fig. 1), were used in this study. They grew in the Experimental Forest Farm at Hunan Academy of Forestry, Hunan, China (28°6.67'N, 113°1.50'E). These two groups of plants were healthy with dark green leaves devoid of any diseases or pest problems. Young leaves were collected from three trees per age group at 08:00 h on 18 April 2017, immediately placed on ice, and taken back to the laboratory. After rinsing with deionized water, they were frozen in liquid nitrogen and stored at -70°C before the following analyses.

Chl content: Leaf Chl contents were analyzed using the method of Arnon (1949). Briefly, frozen leaf samples (0.15 g) were ground in liquid nitrogen and extracted with 80% acetone. After centrifuging, supernatants were analyzed at the absorbance of 645 and 663 nm with a spectrophotometer (Specord 210 Plus, AnalytikJena, Germany), respectively. Chl *a*, *b*, and total (*a*+*b*) were calculated.

Malondialdehyde (MDA) concentration: MDA was tested according to the method described by Heath and Packer (1968). The frozen leaf sample (0.5 g) was ground in liquid nitrogen. After adding 10 mL of 10% trichloroacetic acid, the extract was centrifuged at 4,000 rpm for 10 min. The 2 mL of supernatant was added to 2 mL of 0.6% thiobarbituric acid solution.

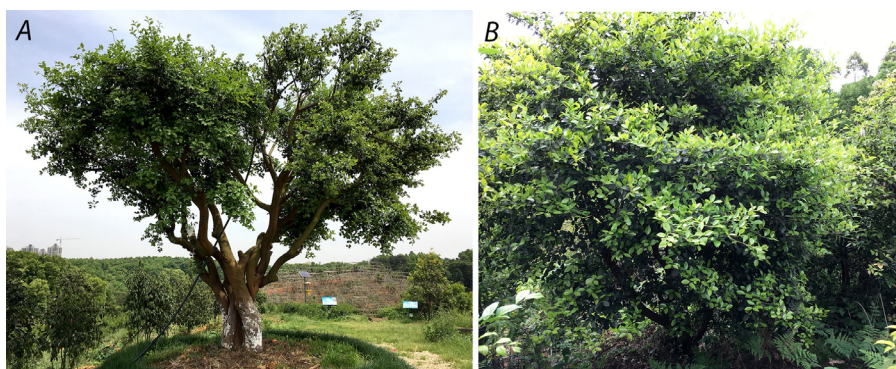


Fig. 1. Growth status of *Camellia oleifera* trees with different ages. (A) A tree over 100 years old was called an ancient tree, and (B) a tree about 30 years old was called a mature tree.

The absorbance of the solution was determined at 450, 532, and 600 nm, respectively, using *Specord 210 Plus*, (*AnalytikJena*, Germany). The concentration of MDA was expressed as $\text{nmol g}^{-1}(\text{FM})$.

Hydrogen peroxide (H_2O_2) concentration: The H_2O_2 contents were analyzed using the method described by Mátaí and Hideg (2017). Briefly, a 0.15-g leaf sample was ground in liquid nitrogen; after adding 1.5 mL of 0.1% trichloroacetic acid, the extract was centrifuged at 12,000 rpm for 20 min. Then 0.25 mL of supernatant was mixed with 0.25 mL of 10 mmol L^{-1} phosphate buffer (pH 7.0). The absorbance was recorded at 390 nm using *Specord 210 Plus* (*AnalytikJena*, Germany). The concentration of H_2O_2 was expressed as $\mu\text{g g}^{-1}(\text{FM})$.

Peroxidase (POD) activity: The POD (EC 1.11.1.7) activity was assayed by the method described by Zieslin and Ben-Zaken (1992). The frozen 0.5-g leaf sample was ground in liquid nitrogen. After adding 5 mL of 0.1 mol L^{-1} phosphate buffer (pH 7.0), the extract was centrifuged at 8,000 rpm for 15 min. The supernatant of 0.25 mL was mixed with 0.25 mL of 10 mmol L^{-1} phosphate buffer (pH 7.0). The absorbance was recorded at 390 nm using *Specord 210 Plus* (*AnalytikJena*, Germany), and then 0.1 mL of supernatant was added to a 3 mL of reaction solution that consisted of 1 mL 0.3% H_2O_2 , 0.95 mL 0.2% guaiacol, 1 mL 50 mmol L^{-1} phosphate buffer (pH 7.0), and 0.05 mL enzyme extract. The changes in absorbance at 470 nm were recorded for 1 min. One unit of POD activity was defined as 1 μg of substrate catalyzed per min per mg of fresh mass.

Superoxide dismutase (SOD) activity: The SOD (EC 1.15.1.1) activity was analyzed according to the method of Sun *et al.* (1988). The frozen leaf sample (0.5 g) was ground in liquid nitrogen. After adding 5 mL of 0.05 mol L^{-1} phosphate buffer (pH 7.8), the extract was centrifuged at 10,000 rpm for 20 min. Then 100 μL of supernatant was mixed with 4 mL of the reaction mixture that consisted of 2.3 mL of 0.05 mol L^{-1} phosphate buffer, 0.4 mL of 130 mmol L^{-1} methionine, 0.4 mL of 750 $\mu\text{mol L}^{-1}$ nitroblue tetrazolium, 0.4 mL of 100 mmol L^{-1} disodium ethylenediaminetetraacetic acid (EDTA-Na_2), 0.1 mL of phosphate buffer, and 0.4 mL of 20 $\mu\text{mol L}^{-1}$ riboflavin. The absorbance was recorded at 560 nm. The absorbance of the control group was taken as a maximum, and the amount of enzyme required to inhibit 50% photochemical

reduction of NBT was calculated as one enzyme activity unit.

Catalase (CAT) activity: The CAT (EC 1.11.1.6) activity was assayed by the method of Zhang *et al.* (2020). The frozen leaf sample (0.5 g) was ground in liquid nitrogen; after adding 5 mL of 0.2 mol L^{-1} phosphate buffer (pH 7.8), the extract was centrifuged at 4,000 rpm for 15 min. The supernatant of 2.5 mL was mixed with 2.5 mL of 10% H_2SO_4 . Then, 0.1 mol L^{-1} potassium permanganate standard solution was continuously added to the mixture until the color turned pink. Finally, CAT activity was calculated. One unit of CAT activity was defined as 1 mg of H_2O_2 catalyzed per min per mg of fresh mass.

Transcriptome sequencing and verification of some differential genes: The total RNA extraction, isolation of poly(A)-containing mRNA, cDNA library construction, sequencing of cDNA library products using *Illumina* as well as data analysis, transcriptome annotation, and identification of relevant genes were performed according to the methods described by Wei *et al.* (2016). After the internal reference gene *ETIF3H* was identified, the expression of related functional genes was verified by real-time quantitative PCR (qRT-PCR). The transcriptomic and qRT-PCR analyses had three biological samples per tree group.

Statistical analysis: Data were statistically analyzed using *SPSS 16.0* (*SPSS Incorporated*, USA). Mean differences between tree groups were separated by *Tukey's* Honest Significant Difference test (HSD) at $P < 0.05$ level.

Results

Physiological characteristics associated with ancient tree leaves: There were significant differences in leaf contents of Chl ($a+b$) and the ratio of Chl a/b between two groups of *C. camellia* trees (Table 1). The Chl ($a+b$) content in leaves of ancient trees was 0.44 $\text{mg g}^{-1}(\text{FM})$, which was higher than 0.26 $\text{mg g}^{-1}(\text{FM})$ of mature trees. Chl a/b in leaves of ancient trees was 10.32 compared to 6.34 of mature trees. MDA concentrations in leaves of ancient trees were higher than mature trees, 8.83 $\text{nmol g}^{-1}(\text{FM})$ vs. 6.51 $\text{nmol g}^{-1}(\text{FM})$, but they were not statistically significant. The H_2O_2 concentration in the leaves of ancient trees [126.77 $\mu\text{g g}^{-1}(\text{FM})$] was significantly higher than that of mature trees [109.43 $\mu\text{g g}^{-1}(\text{FM})$]. Additionally,

Table 1. Leaf chlorophyll contents, MDA and H_2O_2 concentrations, and activities of antioxidant enzymes in ancient and mature tree leaves of *Camellia oleifera*. Data are means \pm standard errors. Different letters after the means indicate significant differences between two groups of trees based on HSD analysis at $P < 0.05$ level. CAT – catalase; Chl – chlorophyll; Chl ($a+b$) – total chlorophyll content; Chl a/b – ratio of Chl a to Chl b ; H_2O_2 – hydrogen peroxide; MDA – malondialdehyde content; POD – peroxidase; SOD – superoxide dismutase; CAT – catalase.

| | Chl ($a+b$) [$\text{mg g}^{-1}(\text{FM})$] | Chl a/b | MDA [$\text{nmol g}^{-1}(\text{FM})$] | H_2O_2 [$\mu\text{g g}^{-1}(\text{FM})$] | SOD [$\text{U g}^{-1} \text{min}^{-1}$] | POD [$\text{U g}^{-1} \text{min}^{-1}$] | CAT [$\mu\text{g g}^{-1} \text{min}^{-1}$] |
|--------------|--|-------------------------------|--|---|--|--|---|
| Ancient tree | 0.44 \pm 0.00 ^a | 10.32 \pm 0.44 ^a | 8.83 \pm 1.25 ^a | 126.77 \pm 4.75 ^a | 671.00 \pm 35.82 ^a | 22.58 \pm 1.01 ^a | 498.42 \pm 34.45 ^a |
| Mature tree | 0.26 \pm 0.01 ^b | 6.34 \pm 0.20 ^b | 6.51 \pm 0.63 ^a | 109.43 \pm 1.99 ^b | 519.94 \pm 17.56 ^b | 14.34 \pm 1.00 ^b | 363.80 \pm 19.40 ^b |

the activities of antioxidant enzymes, SOD, POD, and CAT of ancient tree leaves were significantly higher than those in the mature tree leaves (Table 1).

Chl-related genes differentially expressed in ancient tree leaves: Chl *a/b*-binding protein genes are encoded by the nuclear genome, and their products are the apoproteins of the light-harvesting complex of PSII. These proteins are associated with Chl and xanthophylls and serve as the antenna complex to harvest light and transfer it to photosystems, thus they are critically important for the maintenance of Chl contents and the photosynthetic capability of plants. In this study, ten Chl *a/b*-binding protein genes were identified (Table 2), of which nine were upregulated and only one was downregulated in ancient tree leaves. These upregulated genes may enhance the photosynthetic capacity of ancient *C. oleifera* leaves.

Antioxidant-related genes differentially expressed in ancient tree leaves: SOD, POD, CAT, and ascorbate peroxidase (APX) are common antioxidant enzymes, which are important for clearing up surplus ROS and reducing oxidative stress in plants. This study identified 22 differentially expressed antioxidant-related genes, 15 of them were upregulated and 7 were downregulated in ancient tree leaves (Table 3). Among them, the highest numbers of differential genes occurred in POD family, followed by APX, SOD, and CAT.

Hormone-related genes differentially expressed in ancient tree leaves: Phytohormone contents are closely associated with plant senescence. Usually, auxins and cytokinins (CTK) could prolong plant senescence, whereas abscisic acid (ABA) could accelerate plant senescence. We identified 40 hormone-related genes, which were differentially expressed in leaves of *C. oleifera* trees. Among them, 21 genes were upregulated, and 19 genes were downregulated in ancient tree leaves (Table 4). These genes mainly included ABA, auxin, CKX, and small auxin up RNA (SAUR), wherein the number of auxin-related genes was the highest followed by ABA.

Stress resistance-related genes differentially expressed in ancient tree leaves: Transcription factors, signal transduction factors, stress-resistance genes, and defense-related genes could significantly enhance plant resistance to stresses including senescence. A total of 496 stress resistance-related genes were identified in *C. oleifera* leaves, of which 301 genes were upregulated, and 195 genes were downregulated in ancient tree leaves (Table 5). Stress resistance-related genes mainly included transcription factors of v-Myb avian myeloblastosis viral oncogene homolog (MYB), NAC, MRKY, and zinc finger, signal transduction factors of GTP-binding, receptor protein kinase, resistance protein, and defense-related genes. The number of zinc finger-related genes was the highest, followed by LRR receptor-like serine/threonine-protein kinase, disease-resistance protein, heat-shock protein (HSP), MYB, leucine zipper, basic helix-loop-helix (bHLH), and ethylene-responsive transcription factor (ERF) (Table 5).

Protein degradation-related genes differentially expressed in ancient tree leaves: Protein degradation is an important cause of plant senescence, which usually occurs under the action of several proteases and F-box family protein genes. This study identified 72 functional genes related to protein degradation, which included 17 aspartyl protease genes, 11 cysteine proteinase genes, and 44 F-box family protein genes (Table 6). Among cysteine proteinase, seven genes were upregulated and four were downregulated in ancient tree leaves, whereas more F-box family protein genes and aspartyl protease genes were downregulated in ancient tree leaves.

Verification of selected differentially expressed genes: The genes selected for verification included NAC, F-box, aspartyl protease, and cysteine proteinase. *ETIF3H* was used as the internal reference gene. The qRT-PCR analysis showed that the expressions of *c184012_g3* (F-box), *c178940_g2* (aspartyl protease), and *c163401_g1* (cysteine proteinase) were downregulated in ancient tree

Table 2. Numbers of chlorophyll (Chl) *a/b*-binding protein-related genes differentially expressed in ancient tree leaves.

| Gene category | Main functions | Nr. of differentially expressed genes | Upregulated genes | Downregulated genes |
|---------------------------------|--|---------------------------------------|-------------------|---------------------|
| Chl <i>a/b</i> -binding protein | Maintain chlorophyll content and photosynthetic capacity of plants | 10 | 9 | 1 |

Table 3. Numbers of antioxidant-related genes differentially expressed in ancient tree leaves. APX – ascorbate peroxidase; CAT – catalase; POD – peroxidase; SOD – superoxide dismutase.

| Gene category | Main functions | Nr. of differentially expressed genes | Upregulated genes | Downregulated genes |
|---------------|---|---------------------------------------|-------------------|---------------------|
| SOD | To clear up surplus radicals | 2 | 0 | 2 |
| POD | To clear up ROS and enhance plant stress resistance to stresses | 10 | 9 | 1 |
| CAT | To clear up surplus H ₂ O ₂ | 1 | 0 | 1 |
| APX | To clear up ROS | 9 | 6 | 3 |
| Total | | 22 | 15 | 7 |

Table 4. Numbers of hormone-related genes differentially expressed in ancient tree leaves. ABA – abscisic acid; CKX – cytokinin oxidase; SAUR – small auxin up RNA.

| Gene category | Main functions | Nr. of differentially expressed genes | Upregulated genes | Downregulated genes |
|---------------|--|---------------------------------------|-------------------|---------------------|
| ABA | Inhibition of plant growth, promotion of leaf abscission, and acceleration plant dormancy | 12 | 6 | 6 |
| Auxin | Promotion of lateral and adventitious root generation and adjustment of flowering and sex differentiation, adjustment of fruiting and fruit development, and control of apical dominance | 25 | 13 | 12 |
| CKX | Maintenance or reestablishment of the stability and equilibrium of CTK in plants, enhancement of antioxidation ability of plants | 1 | 1 | 0 |
| SAUR | Maintenance of auxin contents, adjustment of auxin transport, and cell amplification | 2 | 1 | 1 |
| Total | | 40 | 21 | 19 |

Table 5. Numbers of stress resistance related genes differentially expressed in ancient tree leaves. bHLH – basic helix-loop-helix; ERF – ethylene-responsive transcription factor; HSP – heat shock protein; MYB – v-Myb avian myeloblastosis viral oncogene homolog.

| Gene category | Main functions | Nr. of differentially expressed genes | Upregulated genes | Downregulated genes |
|---|---|---------------------------------------|-------------------|---------------------|
| Zinc finger | Defense against drought, high temperature, salt stress, pathogen | 128 | 73 | 55 |
| HSP | Defense against high temperature and drought | 39 | 24 | 15 |
| MYB | Defense against drought, salt, coldness, high-temperature stress, and enhancement of POD activities of plants | 30 | 15 | 15 |
| bHLH | Clearance of ROS and further enhancement of coldness resistance of plants | 31 | 22 | 9 |
| Leucine zipper | Defense against drought and salt stress | 21 | 18 | 3 |
| WRKY | Defense against drought and salt stress | 20 | 18 | 2 |
| NAC | Defense against drought, high temperature, pathogenic bacteria | 10 | 10 | 0 |
| MADS-box | Defense against drought and water stress | 4 | 3 | 1 |
| LRR receptor-like serine/threonine-protein kinase | Defense against drought and salt stress | 74 | 41 | 33 |
| ERF | Enhancement of disease resistance of plants | 21 | 10 | 11 |
| GTP-binding protein | Enhancement of stress resistance and disease resistance of plants | 19 | 6 | 13 |
| Receptor protein kinase | Defense against drought and salt marsh stress | 8 | 5 | 3 |
| Disease resistance protein | Enhancement of disease resistance | 56 | 36 | 20 |
| Pathogenesis-related protein | Defense against various pathogenic bacteria | 13 | 7 | 6 |
| Chitinase | Enhancement of disease resistance of plants | 8 | 6 | 2 |
| Stress protein | Enhancement of resistance of plants to external stress | 14 | 7 | 7 |
| Total | | 496 | 301 | 195 |

leaves but upregulated in mature tree leaves (Table 7). On the other hand, *c167863_g1* (NAC) was upregulated in ancient tree leaves and downregulated in mature tree leaves. In general, the expression patterns of these genes were consistent with the results of transcriptome sequencing, which confirmed the reliability of transcriptome sequencing results.

Discussion

Senescence is the final developmental stage of plant organs and is a process of decay and death of the whole plant. Among them, leaf and flower senescence has been extensively studied (Zhang and Zhou 2013, Dar *et al.* 2021, Guo *et al.* 2021), but information regarding the whole-

Table 6. Numbers of protein degradation-related genes differentially expressed in ancient tree leaves.

| Gene category | Main functions | Nr. of differentially expressed genes | Upregulated genes | Downregulated genes |
|---------------------|--|---------------------------------------|-------------------|---------------------|
| F-box | Involvement in protein degradation of plant cells | 44 | 17 | 27 |
| Aspartyl protease | Involvement in senescence and pathogen-related protein degradation | 17 | 6 | 11 |
| Cysteine proteinase | Involvement in protein hydrolysis | 11 | 7 | 4 |
| Total | | 72 | 30 | 42 |

 Table 7. Verification of selected differentially expressed genes *via* qRT-PCR analysis.

| Selected genes | Expression in ancient tree leaves | Expression in mature tree leaves |
|---|-----------------------------------|----------------------------------|
| <i>c167863_g1</i> (NAC) | 7.25×10^{-4} | 5.46×10^{-5} |
| <i>c178940_g2</i> (aspartyl protease) | 5.70×10^{-3} | 1.26×10^{-1} |
| <i>c163401_g1</i> (cysteine proteinase) | 1.22×10^{-3} | 6.91×10^{-2} |
| <i>c184012_g3</i> (F-box) | 2.09×10^{-3} | 1.13×10^{-1} |

plant senescence is limited (Thomas 2013, Klimešová *et al.* 2015). *C. oleifera* has a lifespan greater than 100 years, representing a model for studying senescence in woody plant species. Nevertheless, leaves are vital organs facilitating photosynthesis (Guo *et al.* 2021), and Chl content was closely related to leaf aging (Chen *et al.* 2015b, Bresson *et al.* 2018). During leaf senescence, some genes involved in photosynthesis would be downregulated, resulting in a decrease in the photosynthetic capability of leaves (Wu *et al.* 2012, Jakhar and Mukherjee 2014). Chl-binding protein genes were downregulated during the senescence process of *Oryza sativa* (Park *et al.* 2007) and *Gossypium* (Kong *et al.* 2013). In this study, the content of Chl (*a+b*) and Chl *a/b* as well as the expression levels of nine Chl *a/b*-binding protein genes were upregulated in ancient *C. oleifera* leaves, which was similar to the results of previous studies (Lu *et al.* 2001, Turfan *et al.* 2020). These data indicate that the ancient *C. oleifera* tree leaves have a high content of Chls, along with the increased expression of Chl *a/b*-binding protein genes, they can maintain plant photosynthesis, support tree growth and development, and delay plant senescence.

Plants produce ROS during their metabolism and growth, but excessive ROS may damage cells and aggravate the aging process of plants. Plants also accumulate ROS during aging and when they experience growth stress. Our results indicate that the contents of MDA and H₂O₂ in leaves of *C. oleifera* increased with the increase of tree age, which was similar to other studies (Turfan *et al.* 2020). However, the activity of SOD, POD, and CAT antioxidant enzymes in the ancient *C. oleifera* leaves was significantly higher, similar to the report of Chang *et al.* (2019). Antioxidant enzymes are critical in the suppression of aging (Liu *et al.* 2022), CAT and POD are specific enzymes that remove H₂O₂, and SOD mainly scavenges superoxide anion radicals. Additionally, antioxidant-related genes play important roles in removing excess ROS in leaves and delaying the aging of *Ginkgo*, *Platycladus*, and other plant species (Li *et al.* 2014, Wang *et al.* 2016, Chang

et al. 2019, Turfan *et al.* 2020, Yan *et al.* 2021). In general, with the increase of tree age, ROS in plant leaves will increase, which leads to senescence. In this study, POD and APX antioxidant-related genes were upregulated in the ancient tree leaves. Thus, with the increased activities of SOD, POD, and CAT and increased expression of POD and APX genes in the ancient tree leaves, the excess ROS could be scavenged in a timely fashion, thereby reducing the ROS stress and delaying plant senescence.

Hormones are vital in the regulation of plant growth and development as well as the plant senescence process. ABA-related genes are upregulated in the senescence process of *Gossypium* (Dong *et al.* 2008, Kong *et al.* 2013), which influenced the senescence of plants by the regulation of growth stress and the induction of ethylene production (Riov *et al.* 1990, Fujii and Zhu 2009). Absciscic acid 8'-hydroxylase, a key functional enzyme, is usually negatively correlated with ABA content in plants (Umezawa *et al.* 2006). Studies have shown that an increase in CTK content can prolong and inhibit leaf senescence by decreasing Rubisco content, increasing antioxidation activities and photosynthetic ability, and enhancing plant resistance to heat and drought stresses (Wingler *et al.* 1998, Umezawa *et al.* 2006, Xu *et al.* 2010, Zhang *et al.* 2010, Le *et al.* 2012). In addition, CKX genes have been found to maintain or re-establish the stability and balance of CTK in plants (Le *et al.* 2012). Our results showed that a CKX-related gene was upregulated in the ancient tree leaves. Additionally, auxin-related genes could prolong plant senescence by inducing auxin contents and adjusting the NAC transcription factors (Kim *et al.* 2011, Zhang and Zhou 2013). In *Gossypium*, an increased expression of auxin-related gene inhibited leaf senescence (Kong *et al.* 2013, Lin *et al.* 2015). In the present study, a SAUR-related gene was upregulated in the ancient *C. oleifera* leaves. The SAUR gene plays an important role in the maintenance of auxin contents by regulating auxin transport and cell amplification (Hou *et al.* 2013, Ren and Gray 2015). With the combined effects of those hormone

genes, hormone contents in *C. oleifera* leaves could be maintained in a balanced fashion, which could promote plant growth and delay senescence.

Abiotic and biotic stresses, such as high or low temperatures, drought, diseases, and pests were closely related to plant senescence (Kurepa *et al.* 2009, Ho 2015). Due to many external growth stresses, stress-related transcription factors would be induced. In *Arabidopsis thaliana* (Yang *et al.* 2011), *Gossypium* (Kong *et al.* 2013), and *Oryza sativa* (Gao *et al.* 2009), increased expression of NAC transcription factors prolonged plant senescence process and improved drought and salt stresses. The MYB transcription factor has been demonstrated to enhance the resistance to drought, salt, and low or high temperatures in *Lycopersicon esculentum* (Vannini *et al.* 2007), *A. thaliana* (Dai *et al.* 2007, Ding *et al.* 2009, Guo and Gan 2011), and *Salicornia brachiata* (Shukla *et al.* 2015). In *O. sativa* (Pandey and Somssich 2009) and *A. thaliana* (Wan *et al.* 2018), WRKY has been shown to influence leaf senescence by enhancing the resistance to drought, high temperature, and pathogenic bacteria. The bHLH transcription factor has been reported to enhance POD activity, reduce ROS, and further increase trifoliate orange tolerance to low temperatures (Huang *et al.* 2013). In *Brassica rapa* (Saha *et al.* 2015) and *L. esculentum* (Yin *et al.* 2017), the elevated expression of the MADS-box gene enhanced plant resistance to drought and salt stresses. The HSPs transcription factor is known to strengthen plant tolerance to high temperatures and drought (Mishra *et al.* 2002, Jacob *et al.* 2017). In *A. thaliana* (Zang *et al.* 2016) and *Populus trichocarpa* (Liu *et al.* 2015), it has been found that the expression of the functional gene of zinc finger protein enhanced the stress resistance of plants to drought, high temperature, salt stress, and pathogen attack by the adjustment of antioxidant activities of plants (Ordiz *et al.* 2002, Gupta *et al.* 2012). The leucine zipper transcription factor SlbZIP1 could regulate ABA biosynthesis and enhance salt and drought resistance of *L. esculentum* (Zhu *et al.* 2018). Many transcription factors are implicated in plant disease resistance (Takenaka *et al.* 2009, Kumar *et al.* 2018). The GTP-binding protein plays an important role in cell signal transduction and the improvement of plant resistance to diseases (Jeworutzki *et al.* 2010, Lee *et al.* 2017). The LRR receptor-like serine/threonine protein kinase (FLS2) is involved in plant-pathogen interaction and disease resistance (Chinchilla *et al.* 2007, Goff and Ramonell 2007, Ho 2015, Ye *et al.* 2017). Ethylene-responsive transcription factors (ERF) are not only involved in plant senescence but also participate in the regulation of plant stress response (Singh *et al.* 2002, Koyama *et al.* 2013, Mase *et al.* 2013). In the present study, 496 transcription factors were identified, of which 301 were upregulated and 195 were downregulated in ancient *C. oleifera* tree leaves (Table 5), which is similar to the other reports (Wang *et al.* 2020, Batalova and Krutovsky 2023). The upregulation of most transcription factors suggests that ancient *C. oleifera* trees might have an increased ability to tolerate environmental stresses, thus suppressing the plant senescence process.

Plant senescence is accompanied by protein degradation (Jakhar and Mukherjee 2014). Cysteine proteinase is regarded as the most abundant enzyme related to leaf senescence (Diaz-Mendoza *et al.* 2016) and protein hydrolysis (Cruz de Carvalho *et al.* 2001). The expression of aspartic protease was implicated in petal senescence and pathogen-related protein degradation (Cruz de Carvalho *et al.* 2001) and increased senescence in *Glycine max* (Cruz de Carvalho *et al.* 2001) and *A. thaliana* (Espinoza *et al.* 2007). F-box gene was one of the largest gene families that took part in cell protein degradation in chickpea (Gupta *et al.* 2015) and *O. sativa* (Chen *et al.* 2013). With the degradation of proteins, the senescence process of plants would be exacerbated. In ancient *C. oleifera* leaves, the overall expression of functional genes related to protein degradation were downregulated. Our analysis showed that the expression of 11 aspartyl protease genes, four cysteine proteinase genes, and 27 F-box family protein genes were downregulated in ancient *C. oleifera* leaves, which was similar to the report of Guo *et al.* (2021). These results suggested that the decrease in expression of these functional genes may help reduce protein degradation and delay the senescence of the ancient *C. oleifera* trees.

Conclusions: Whole-plant senescence remains largely unexplored thus far. To gain information on the long lifespan of *C. oleifera*, this study compared leaf physiological and molecular parameters of ancient trees with mature trees. Our results showed that the leaves of the ancient trees had higher Chl contents and higher activities of antioxidant enzymes, which could help effectively remove ROS. Additionally, most genes related to Chl *a/b*-binding proteins, POD, APX, auxin, transcription factors, signal transduction, and defense responses were upregulated. On the other hand, genes related to aspartyl protease, cysteine proteinase, and F-box family proteins were largely downregulated. The differentially regulated gene expression could enhance ancient plants to resist abiotic and biotic stresses and reduce protein degradation. Taken together, the increased Chl contents and antioxidant enzyme activities, upregulation of stress-related gene expression, and downregulation of protein degradation gene expression could enable the ancient *C. oleifera* trees to continuously maintain a state of rejuvenation, thus expanding their longevity.

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