

Article

PsAOS3 Enhances High-Temperature Tolerance of Tree Peony by Promoting JA Accumulation

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Abstract

High-temperature stress severely restricts plant growth and development by inducing oxidative damage and photosynthetic inhibition. Allene oxide synthase (AOS) is a key enzyme in jasmonic acid (JA) biosynthesis, yet its role in the high-temperature stress response of woody ornamental plants remains largely uncharacterized. In this study, *PsAOS3* was isolated and characterized from tree peony (*Paeonia suffruticosa* Andr.), an economically important ornamental species susceptible to high-temperature stress. *PsAOS3* was localized to the plasma membrane and was strongly induced by high-temperature stress. Functional analyses using virus induced gene silencing in tree peony showed that silencing of *PsAOS3* accelerated chlorophyll loss, increased reactive oxygen species (ROS) accumulation, impaired photosystem II efficiency and membrane integrity, and reduced antioxidant enzymes and endogenous JA contents under high-temperature stress. Conversely, overexpression of *PsAOS3* in tobacco (*Nicotiana tabacum* L.) alleviated chlorophyll loss, decreased ROS accumulation, improved photosystem II efficiency and membrane integrity, and elevated antioxidant enzymes and endogenous JA contents under high-temperature stress. Collectively, this study reveals a potential role of *PsAOS3* in regulating high-temperature responses in tree peony.

Keywords: tree peony; *PsAOS3*; JA accumulation; high-temperature stress; antioxidant defense

1. Introduction

Increasingly frequent and prolonged high-temperature events due to global warming have emerged as a major abiotic constraint on plant growth, ecosystem stability, and agricultural and horticultural productivity [1,2]. High-temperature stress damages cellular membranes, promotes membrane lipid peroxidation, suppresses key photosynthetic enzymes, and disrupts chlorophyll biosynthesis and turnover. It also causes excessive production of reactive oxygen species (ROS), disturbs cellular redox homeostasis, and consequently impairs growth and physiological metabolism, potentially resulting in plant death [3,4]. China is particularly vulnerable to this trend, as extreme high-temperature events now occur frequently in many regions. In the middle and lower reaches of the Yangtze River, persistent summer high temperatures often coincide with critical stages of crop development, creating a substantial threat to horticultural production.

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Tree peony (*Paeonia suffruticosa* Andr.), traditionally celebrated in China as the “king of flowers” has considerable ornamental, cultural, and economic importance [5,6]. Its symbolism of prosperity and auspiciousness has long resonated with aspirations for a better life. With socioeconomic development and rising living standards, demand for tree peony has expanded beyond its traditional production areas, encouraging cultivation in a broader range of regions [7]. The species originated in the temperate areas of northern China and is adapted to relatively cool, dry conditions. In recent years, the strong consumer market, developed economy, and demand for urban landscaping in the middle and lower Yangtze River basin have made this region an important destination for the southward expansion of tree-peony cultivation [8]. Improvements in breeding and cultivation technology have further supported its large-scale introduction. Nevertheless, summer high temperatures frequently cause progressive deterioration, commonly summarized as “flowers in the first year, leaves in the second, and bare stems in the third”. High-temperature stress therefore remains a major obstacle to the establishment of tree peony in this region and to the sustainable development of the industry [9].

Several approaches have been used to reduce high-temperature related growth inhibition, including exogenous hormone application [10,11], modification of the cultivation environment [12,13], selection of high-temperature-tolerant germplasm, and genetic engineering [14,15]. Hormonal treatments are widely used in plant-stress studies because they are straightforward and often effective. Jasmonic acid (JA) is an important growth regulator involved in not only development [16], flowering, and fruiting [17] but also in responses to abiotic and biotic challenges, including high temperatures [18], drought [19], herbivory [20], and disease [21]. JA can improve stress tolerance by maintaining redox homeostasis, enhancing antioxidant capacity, and stabilizing the photosynthetic apparatus. Both external application and increased endogenous production of JA have been associated with greater thermotolerance. In rice (*Oryza sativa* L.), foliar methyl jasmonate (MeJA) mitigates high-temperature-induced losses in differentiated spikelet number and yield by improving carbon and nitrogen metabolism [22]. In cucumber (*Cucumis sativus* L.), MeJA reduces leaf wilting, electrolyte leakage, MDA accumulation, and ROS levels while increasing proline content under high-temperature stress [23]. High-temperature-tolerant rose (*Rosa hybrida*) cultivars also maintain higher JA and JA-Ile concentrations than high-temperature-sensitive cultivars under both control and high-temperature conditions [24]. Together, these findings support JA as a central signal in plant thermotolerance. Its contribution to the high-temperature response of tree peony, however, remains undetermined.

In the JA biosynthetic pathway, allene oxide synthase (AOS) is a key enzyme responsible for converting 13-hydroperoxy-linolenic acid (13-HPOT) into unstable allene oxides, which are subsequently converted into cyclopentenone compounds, leading to JA biosynthesis. Plant AOS proteins belong to the cytochrome P450 CYP74 family, which consists of several subfamilies, including CYP74A, CYP74B, CYP74C, and CYP74D. However, members of the CYP74 family exhibit functional diversification, and not all CYP74 proteins participate in JA biosynthesis. Among them, CYP74A-type AOS proteins have been widely characterized as key enzymes involved in the JA biosynthetic pathway, whereas certain CYP74C members, such as tomato (*Solanum lycopersicum*) LeAOS3, have also been reported to exhibit AOS activity. Other CYP74 members, such as hydroperoxide lyase (HPL), are involved in alternative oxylipin pathways [25–27]. Therefore, identification and functional characterization of specific AOS homologs are necessary to understand their contribution to JA-mediated stress responses. This enzyme catalyzes a key rate-limiting step in JA biosynthesis, and its abundance and activity can strongly influence endogenous JA accumulation [25]. Genetic studies have shown that overexpressing *OsAOS2* in rice or peach (*Prunus persica*) and *PpAOS3* in *Arabidopsis* raises endogenous JA levels [28,29]. Evidence also links AOS

to plant high-temperature responses. In Arabidopsis, combined high-light and high-temperature treatment markedly induces *AtAOS*, whereas *ataos* mutants are hypersensitive to this stress combination; exogenous JA partially rescues the phenotype [30]. Similarly, *SLAOS1* overexpression in tomato promotes JA accumulation, limits ROS overproduction, and increases thermotolerance, while *SLAOS1* knockout has the opposite effects [31]. Despite these observations, direct functional evidence connecting *AOS* genes with thermotolerance is still limited, particularly for woody ornamental species.

Research on the JA-mediated mitigation of high-temperature injury has largely focused on herbaceous species [32–34], and relatively little is known about the use of exogenous JA or the manipulation of endogenous JA biosynthesis to improve high-temperature tolerance in woody ornamentals. In a previous study, exogenous MeJA treatment was found to enhance high-temperature tolerance in tree peony, which prompted us to investigate the underlying endogenous regulatory mechanisms. Here, we identify *PsAOS3*, a candidate gene in the JA-accumulation pathway, and evaluate its function through virus-induced gene silencing (VIGS) in tree peony and heterologous overexpression in tobacco. The findings provide mechanistic insight into JA-mediated thermotolerance and identify *PsAOS3* as a candidate gene for further functional studies in tree peony.

2. Materials and Methods

2.1. Plant Materials and Treatment

Potted tree peonies were grown in a sterilized substrate consisting of soil, vermiculite, and perlite at a ratio of 1:1:1 (*v/v/v*). Plants of the cultivar ‘Hua Wang’ bearing 3–5 buds were used for gene cloning and high-temperature-responsive expression analysis. Samples used for gene cloning were collected in April 2022. For expression analysis, plants were exposed to 40 °C for 72 h, while control plants were maintained at 20 °C under otherwise identical conditions. Samples were collected in May 2022 after completion of the treatment.

Two-year-old seedlings of the tree peony cultivar ‘Rou Furong’ were used for the virus-induced gene silencing (VIGS) assay. Plants inoculated with the *PsAOS3*-silencing constructs (pTRV-L1 or pTRV-L2) and those inoculated with the corresponding empty pTRV vector were simultaneously subjected to high-temperature treatment at 40 °C during the 16-h light period and 35 °C during the 8-h dark period. Samples were collected in April 2023 after 72 h of treatment, when the *PsAOS3*-silenced plants exhibited evident high-temperature stress symptoms, including leaf wilting and yellowing. Empty-vector control plants subjected to the same treatment were sampled simultaneously.

PsAOS3-overexpressing transgenic tobacco and wild-type (WT) plants were simultaneously exposed to the same day/night high-temperature regime described above. Samples were collected in September 2024 after 96 h of treatment, when WT plants displayed visible high-temperature-induced symptoms, including wilting and yellowing. The corresponding transgenic plants were sampled simultaneously for subsequent physiological and biochemical analyses.

All high-temperature treatments were conducted in controlled-environment growth chambers at 60–70% relative humidity and a light intensity of approximately 30,000 lux. All experiments were performed using three independent biological replicates. Samples from the different experimental groups were collected simultaneously according to the treatment durations and phenotypic criteria described above.

2.2. Cloning and Characterization of *PsAOS3*

Total RNA was extracted from tree peony leaves and reverse-transcribed using EasyScript RT/RI Enzyme Mix (TransGen, Beijing, China). Gene-specific primers designed from the tree peony transcriptome (Table S1) were used to amplify the complete *PsAOS3*

coding sequence (CDS). The purified amplicon was ligated into a cloning vector (Vazyme, Nanjing, China), propagated in *Escherichia coli*, and verified by Sanger sequencing (Sangon, Shanghai, China). The deduced PsAOS3 protein sequence was used as a BLASTP query against the NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 3 August 2022) to retrieve homologs. Protein sequences were aligned with ClustalW in MEGA 7.0, and a neighbor-joining phylogenetic tree was then constructed.

2.3. Gene Expression Pattern Analysis

Leaf RNA was extracted as described above, and first-strand cDNA was synthesized according to the manufacturer's protocol (TransGen, Beijing, China). RT-qPCR was performed using SYBR Green chemistry (TransGen) on a Bio-Rad real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) with the gene-specific primers listed in Table S1. Tree peony beta-tubulin and tobacco Actin served as the respective internal reference genes.

2.4. Subcellular Localization Observation

Subcellular localization of PsAOS3 was first predicted using the online tool Plant-mPLOC (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>, accessed on 4 November 2022). For subcellular localization, the PsAOS3 CDS was inserted downstream of the CaMV 35S promoter in pCambia2300-GFP to generate 35S:PsAOS3-GFP (Table S1). The fusion construct and empty-vector control were separately introduced into *Agrobacterium tumefaciens* GV3101 and infiltrated into leaves of 40-day-old *N. benthamiana*. Fluorescence was examined 48 h after infiltration using a Zeiss LSM 880NLO confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). AtPIP2A was selected as a well-established plasma membrane marker based on previous reports [15] to experimentally verify the predicted plasma membrane localization. PsAOS3-GFP was also co-expressed with the reported plasma-membrane marker mCherry-AtPIP2A (AT3G53420). GFP was excited at 488 nm and detected at 494–563 nm (gain = 100). For mCherry imaging, excitation was set to 561 nm and emission was collected at 567–635 nm (gain = 100). Chlorophyll autofluorescence was detected in the far-red channel (excitation 640 nm, emission 650–750 nm) to distinguish chloroplast signals.

2.5. Gene Silencing in Tree Peony

PsAOS3 was silenced in tree peony using the pTRV-based VIGS system. A gene-specific, non-conserved PsAOS3 fragment was inserted into pTRV2. *A. tumefaciens* GV3101 cultures carrying pTRV1, empty pTRV2, or recombinant pTRV2 were resuspended in induction buffer and incubated at 37 °C for 1 h. Each pTRV2 culture was then combined with pTRV1 at a 1:1 (v/v) ratio. Selected plants were immersed in the bacterial suspension and vacuum-infiltrated at 0.45 MPa for 20 min. After infiltration, plants were washed and transplanted into a 1:1:1 mixture of garden soil, vermiculite, and perlite. Transformation and silencing efficiency were assessed by PCR and RT-qPCR, respectively, using the primers in Table S1.

2.6. Gene Overexpression in Tobacco

The full-length PsAOS3 CDS was inserted into pCambia1301 to produce pCambia1301-PsAOS3, which was transferred into *A. tumefaciens* GV3101 by the freeze-thaw method. Tobacco 'K326' leaf explants were co-cultured with GV3101 carrying the recombinant vector, and transgenic plants were recovered through regeneration and differentiation. Putative transformants were confirmed by PCR using the gene-specific primers listed in Table S1. The transgenic tobacco lines used in this study were T3 generation homozygous lines.

2.7. Stress Physiological Index Measurement

Leaf b^* values were recorded with a handheld RM200QC spectrophotometer (X-Rite, Incorporated, Grand Rapids, MI, USA), and SPAD values were measured using a SPAD-502 chlorophyll meter (Konica Minolta, Tokyo, Japan). Relative electrical conductivity (REC) was determined following Yang et al. [35], whereas malondialdehyde (MDA) content was measured with a commercial kit (Suzhou Comin Biotechnology Co., Ltd., Suzhou, China).

To assess relative ROS accumulation, hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$) levels were detected by 3,3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining, respectively, as qualitative visualization methods. Leaves were submerged in the appropriate staining solution and incubated in darkness for 2–6 h, until brown DAB precipitates or blue NBT products became visible at sites of accumulation. Excess stain was removed by washing the samples 3–5 times with distilled water. Chlorophyll was then cleared by immersing the leaves in 95% ethanol in a boiling-water bath for 10–30 min. After a final 30-min soak in clean water, the leaves were photographed.

Leaf tissue (0.5 g) was ground to a fine powder in liquid nitrogen and homogenized in ice-cold 50 mM phosphate buffer (pH 7.8). Following centrifugation at $10,000\times g$ for 15 min at 4 °C, the supernatant was used to determine superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities with SOD-2-Y, POD-2-Y, and CAT-2-Y kits, respectively (Suzhou Comin Biotechnology Co., Ltd., China).

Endogenous jasmonic acid (JA) levels were determined using a commercial Plant JA ELISA Kit (SP29738; Saipai Biotechnology Co., Ltd., Guangzhou, China) according to the manufacturer's instructions. Briefly, 0.1 g of fresh leaf tissue was ground in liquid nitrogen and homogenized in 1 mL of $1\times$ phosphate-buffered saline (PBS). The homogenate was centrifuged at 12,000 rpm for 1 min at 4 °C, and the resulting supernatant was collected and directly subjected to ELISA analysis without further dilution.

A calibration curve was generated using JA standards at 0, 125, 250, 500, 1000, and 2000 pmol L^{-1} . For each sample well, 10 μL of the supernatant and 40 μL of sample diluent were added, whereas each standard well received 50 μL of the corresponding standard. Subsequently, 100 μL of horseradish peroxidase-conjugated reagent was added to each well, and the plate was incubated at 37 °C for 60 min. After washing five times with 400 μL of diluted wash buffer, 50 μL each of chromogenic solutions A and B were added. The plate was incubated at 37 °C for 15 min in the dark, and the reaction was terminated by adding 50 μL of stop solution. Absorbance was measured at 450 nm within 15 min using a microplate reader. JA concentrations were calculated from the standard curve and normalized to the fresh weight of the leaf samples, with the results expressed as $ng\ g^{-1}$ fresh weight (FW). Three independent biological replicates were analyzed for each treatment.

Gas-exchange measurements were taken at 08:00 with an LI-6400 portable photosynthesis system (LI-Cor, Lincoln, NE, USA). A 2×3 cm leaf chamber fitted with a red-blue LED source was used at a photosynthetic photon flux density of $1000\ mol\ m^{-2}\ s^{-1}$. Net photosynthetic rate (P_n) and stomatal conductance (G_s) were recorded. Chlorophyll fluorescence was measured with a fluorometer (Heinz Walz GmbH, 91090 Effeltrich, Germany) after 2 h of dark adaptation. The maximum quantum efficiency of photosystem II (F_v/F_m) and non-photochemical quenching coefficient (q_N) were determined as described previously [36,37].

2.8. Statistical Analysis

Values are reported as the mean $\pm$ SD of at least three independent biological replicates. Differences among treatments were evaluated in SPSS Statistics 25.0 (SPSS, Chicago, IL, USA) by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Figures were generated using GraphPad Prism 8.

3. Results

3.1. PsAOS3 Screening and Sequence Analysis

To identify candidate AOS genes involved in high-temperature stress responses, we first examined five nonredundant AOSs in our previously generated transcriptome of tree peony exposed to 40 °C (BioProject accession PRJNA1429858). Comparison of their FPKM profiles revealed that Unigene6171 exhibited the strongest response to high-temperature stress (Figure 1a). This expression pattern was independently confirmed by RT-qPCR, which showed that transcript levels of Unigene6171 were approximately 15-fold higher after 72 h at 40 °C than at 20 °C (Figure 1b). A BLASTP search against the NCBI database revealed that the deduced amino acid sequence of Unigene6171 shared high homology with AOS3 proteins from various plant species; accordingly, the gene was designated PsAOS3 (Figure 1c). To further classify PsAOS3 within the CYP74 family, we performed a phylogenetic analysis including members of the CYP74A, CYP74B, CYP74C, and CYP74D subfamilies [27]. This analysis placed PsAOS3 in the CYP74C subfamily, together with other AOS3 orthologs (Figure S1). Although CYP74C orthologs are not ubiquitous in plants, the inclusion of PsAOS3 in this clade indicates that tree peony would possess a functional CYP74C AOS. Alignment of the PsAOS3 amino acid sequence further showed that it contains the characteristic AOS motifs, including the I-helix, ETLR motif, and heme-binding region (Figure 1d).

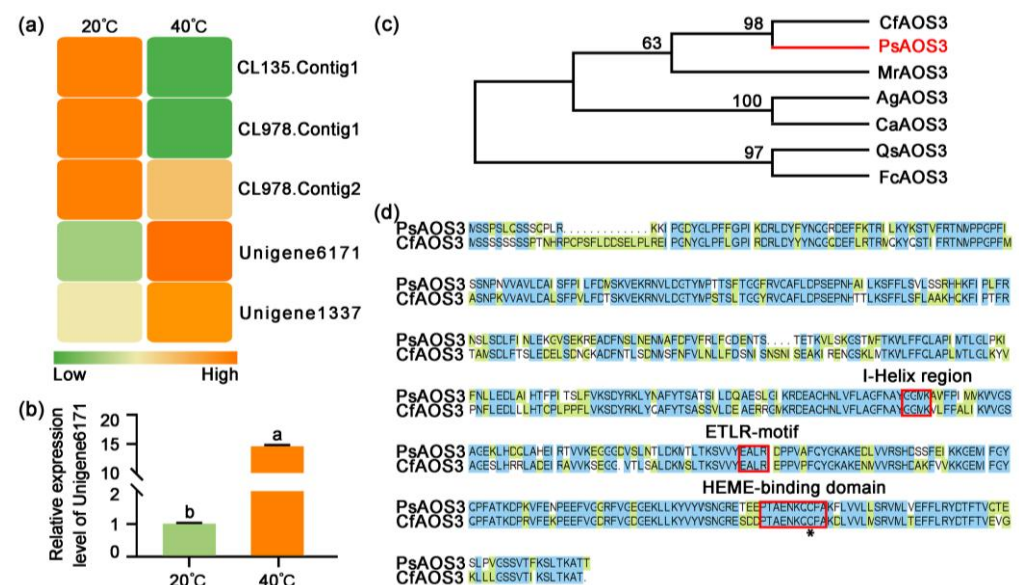


Figure 1. Characterization of PsAOS3. (a) Expression patterns of five nonredundant AOS genes from tree peony transcriptome based on the FPKM values. (b) Expression patterns of Unigene6171 from tree peony using RT-qPCR. Tree peony was treated with 20 °C and 40 °C for 72 h. (c) Phylogenetic analysis of AOS3 from tree peony and other plants (CfAOS3: *Cornus florida*, XP_059644081.1; MrAOS3: *Morella rubra*, KAB1207208.1; AgAOS3: *Alnus glutinosa*, XP_062176352.1; CaAOS3: *Corylus avellana*, XP_059428255.1; QsAOS3: *Quercus suber*, XP_023881556.2; FcAOS3: *Fagus crenata*, GMY05899.1). (d) Sequence alignment of PsAOS3 and CfAOS3. The I-Helix region, ETLR-motif, and HEME-binding domain are indicated by red boxes, respectively. The cytochrome P450 binding domain is marked by an asterisk. The values represent the means $\pm$ SDs, and different letters indicate significant differences ($p < 0.05$).

3.2. Subcellular Localization of PsAOS3

Subcellular localization of PsAOS3 was first predicted using the online tool Plant-mPLOC, which suggested possible targeting to both chloroplasts and the plasma membrane. To experimentally verify this prediction, the subcellular distribution of PsAOS3 was assessed by co-expressing 35S:PsAOS3-GFP with the mCherry-tagged plasma membrane marker AtPIP2A in *N. benthamiana* epidermal cells. The PsAOS3-GFP signal largely coincided with AtPIP2A-mCherry fluorescence, producing a clear yellow signal in the merged images. In contrast, the PsAOS3-GFP signal did not overlap with chlorophyll autofluorescence (Figure 2). This colocalization indicated that PsAOS3 was localized to the plasma membrane.

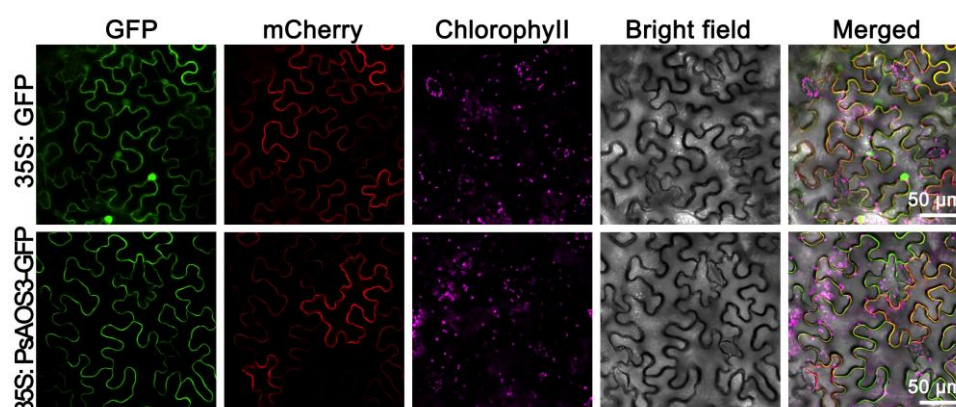


Figure 2. Subcellular localization of PsAOS3. Constructs of p35S:PsAOS3-GFP or p35S:GFP combined with p35S:AtPIP2A-mCherry (Plasma membrane localization, *Arabidopsis thaliana* AtPIP2A, AT3G53420) were transferred to *Nicotiana benthamiana* leaf epidermal cells. The GFP, mCherry and chlorophyll signals are excited and visualized with 488 nm, 561 nm and 640 nm wavelengths, respectively.

3.3. Silencing PsAOS3 Reduced the High-Temperature Tolerance of Tree Peony Through Inhibiting JA Accumulation

To determine whether PsAOS3 contributes to tree peony thermotolerance, two PsAOS3-silenced lines (pTRV-L1 and pTRV-L2) were generated by VIGS, with plants carrying the empty pTRV vector serving as controls. Detection of the expected TRV1- and TRV2-specific amplicons verified successful transformation (Figure S2a). RT-qPCR further showed that PsAOS3 transcript abundance was reduced by 37.6% in pTRV-L1 and 44.1% in pTRV-L2 (Figure S2b). The silenced and control plants were phenotypically similar under normal conditions. After 72 h of high-temperature stress (40 °C/35 °C; 16 h light/8 h dark), however, both silenced lines wilted more severely than the controls (Figure 3a). They also exhibited b^* values (a yellow–blue color component indicative of leaf yellowing) approximately 20% higher and SPAD values (a non-destructive indicator of relative chlorophyll content) 12% lower than those of the control plants (Figure 3b), indicating greater leaf yellowing and chlorophyll loss. We then assessed indices of high-temperature induced physiological injury. High-temperature stress increased both REC and MDA content. Under high-temperature stress, the values measured in the PsAOS3-silenced lines were 1.8- and 2.1-fold, respectively, those of the controls (Figure 4a), consistent with more extensive membrane injury and lipid peroxidation. DAB and NBT staining likewise revealed greater ROS accumulation in silenced leaves than in control leaves after high-temperature exposure (Figure 4b). Antioxidant capacity was also compromised: compared with the controls, the silenced lines showed reductions of 18%, 30%, and 20% in SOD, POD, and CAT activities, respectively (Figure 5a). Their JA content was approximately 40% lower than that of the controls under high-temperature stress (Figure

5b). In parallel, stomatal conductance (G_s) and the net photosynthetic rate (P_n) decreased by 40% and 35%, respectively. The maximum quantum efficiency of PSII (F_v/F_m) and the non-photochemical quenching coefficient (q_N) were used to evaluate PSII performance. Compared with the control, F_v/F_m decreased by 18%, while q_N increased by 23.7% in the silenced lines (Figure 5c,d). These changes indicate impaired PSII function and photosynthetic performance. Collectively, suppression of *PsAOS3* lowered JA accumulation and increased high-temperature-induced physiological damage in tree peony.

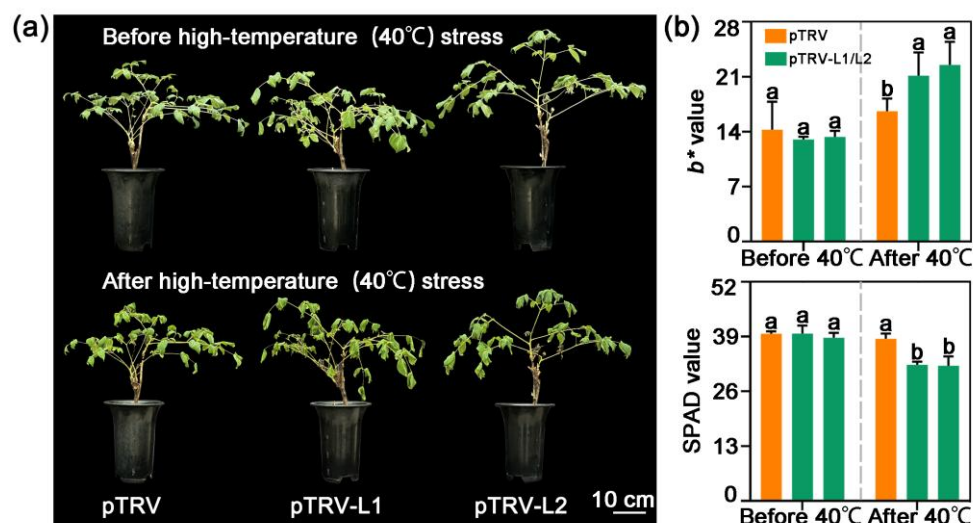


Figure 3. Functional analysis of *PsAOS3* in tree peony using virus-induced gene silencing (VIGS). (a) Phenotypes of the *PsAOS3*-silenced plants (pTRV-L1, pTRV-L2) and the pTRV empty vector-transformed plants (pTRV) before and after high-temperature stress (40 °C/35 °C, 16 h light/8 h dark) for 72 h. (b) b^* and SPAD values of the corresponding plants under the same conditions. The values represent the means $\pm$ SDs, and different letters indicate significant differences ($p < 0.05$).

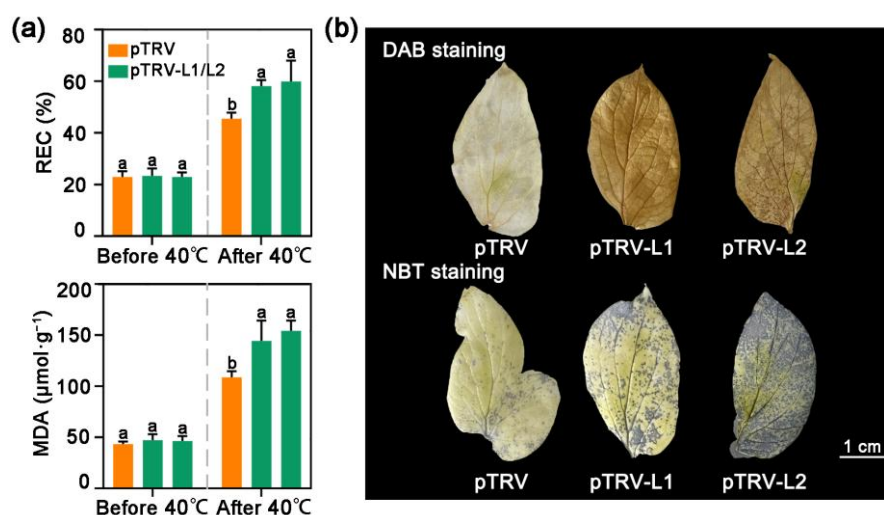


Figure 4. Stress physiological indices of the *PsAOS3*-silenced plants (pTRV-L1, pTRV-L2) and pTRV empty vector-transformed plants (pTRV) before and after high-temperature stress (40 °C/35 °C, 16 h light/8 h dark) for 72 h. (a) REC and MDA content. (b) ROS accumulation. H_2O_2 accumulation is detected by DAB staining. $\text{O}_2^{\cdot -}$ accumulation is detected by NBT staining. The values represent the means $\pm$ SDs, and different letters indicate significant differences ($p < 0.05$).

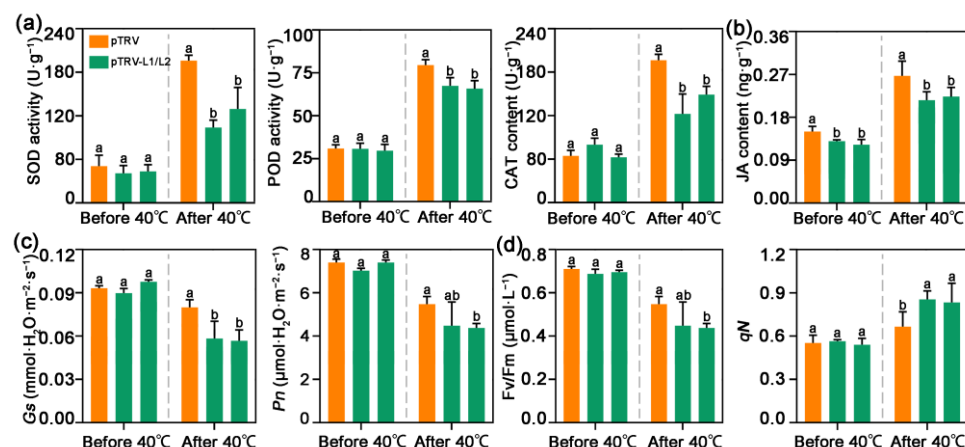


Figure 5. Antioxidant enzyme activities, jasmonic acid contents, photosynthetic characteristics and chlorophyll fluorescence parameters of *PsAOS3*-silenced plants (pTRV-L1, pTRV-L2) and pTRV empty vector-transformed plants (pTRV) before and after high-temperature stress (40 °C/35 °C, 16 h light/8 h dark) for 72 h. (a) Antioxidant enzyme activities. (b) Jasmonic acid contents. (c) Photosynthetic characteristics. (d) Chlorophyll fluorescence parameters. The values represent the means $\pm$ SDs, and different letters indicate significant differences ($p < 0.05$).

3.4. Overexpression of *PsAOS3* Improved the High-Temperature Tolerance of Tobacco Through Increasing JA Accumulation

To further evaluate *PsAOS3* function, two tobacco lines overexpressing *PsAOS3* (OE-L1 and OE-L2) were generated and compared with WT plants. PCR and RT-qPCR analysis confirmed the presence of the transgene in both lines (Figure S3). Under normal growth conditions, the transgenic and WT plants displayed comparable phenotypes (Figure 6a), and their leaf b^* and SPAD values did not differ significantly (Figure 6b). Following high-temperature stress for 96 h (40 °C/35 °C; 16 h light/8 h dark), OE-L1 and OE-L2 retained lower b^* values and higher SPAD values than WT plants, indicating reduced leaf yellowing and chlorophyll loss. High-temperature exposure increased membrane lipid peroxidation and ROS accumulation in tobacco, but both REC and MDA contents were significantly lower in the transgenic lines than in WT plants (Figure 7a). DAB and NBT staining also showed less H₂O₂ and O₂⁻ accumulation in OE-L1 and OE-L2 leaves (Figure 7b), consistent with reduced oxidative injury. Although high-temperature stress increased SOD, POD, and CAT activities in all plants, the increases were significantly greater in the overexpression lines (Figure 8a). JA content determination showed that the overexpression lines consistently maintained significantly higher JA levels than WT plants under both normal and high-temperature conditions, with the difference being approximately 1.9-fold under high-temperature stress. (Figure 8b). Both P_n and G_s remained significantly higher in OE-L1 and OE-L2 following high-temperature stress (Figure 8c). The overexpression lines also maintained higher Fv/Fm and lower qN values than WT plants (Figure 8d), indicating better preservation of PSII photochemical function and less non-photochemical energy dissipation. Thus, *PsAOS3* overexpression promoted JA accumulation and enhanced the thermotolerance of tobacco.

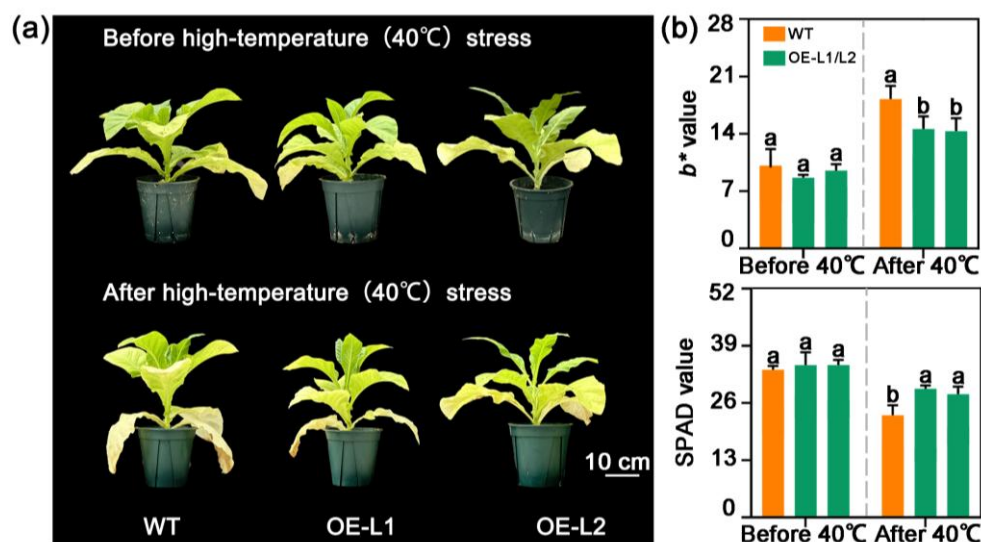


Figure 6. Functional analysis of overexpressing *PsAOS3* in tobacco. (a) Phenotypes of the wild-type (WT) and transgenic tobacco lines overexpressing *PsAOS3* (OE-L1, OE-L2) before and after high-temperature stress (40 °C/35 °C 16 h light/8 h dark) for 96 h. (b) b^* and SPAD values of the corresponding plants under the same conditions. The values represent the means $\pm$ SDs, and different letters indicate significant differences ($p < 0.05$).

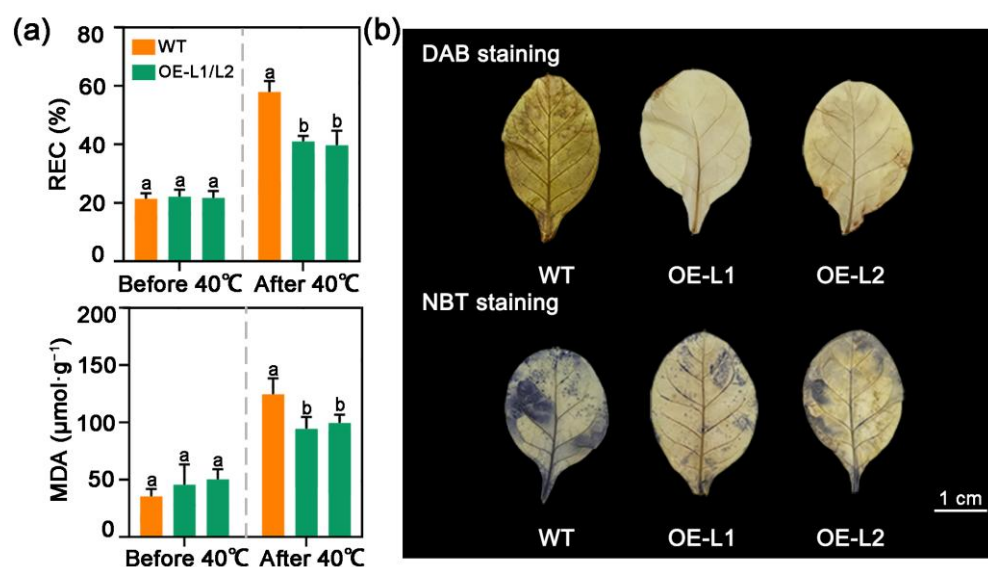


Figure 7. Stress physiological indices of the wild-type (WT) and transgenic tobacco lines overexpressing *PsAOS3* (OE-L1, OE-L2) before and after high-temperature stress (40 °C/35 °C 16 h light/8 h dark) for 96 h. (a) REC and MDA content. (b) ROS accumulation. H_2O_2 accumulation is detected by DAB staining. $\text{O}_2^{\cdot-}$ accumulation is detected by NBT staining. The values represent the means $\pm$ SDs, and different letters indicate significant differences ($p < 0.05$).

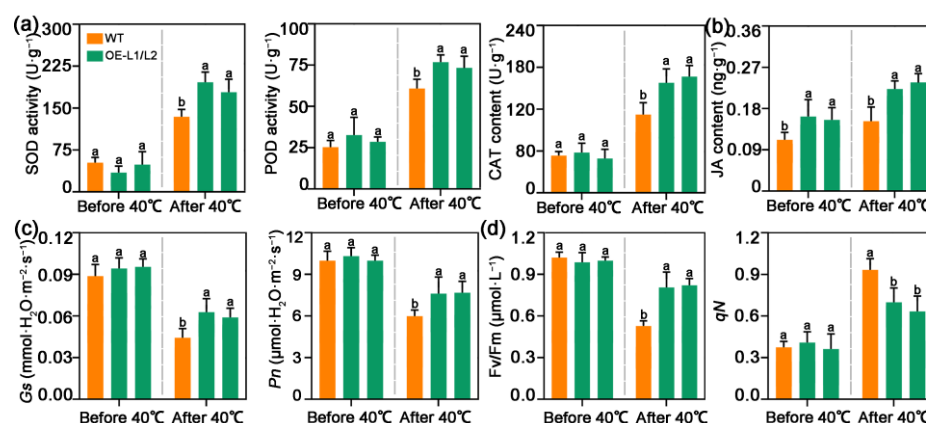


Figure 8. Antioxidant enzyme activities, jasmonic acid contents, photosynthetic characteristics and chlorophyll fluorescence parameters of the wild-type (WT) and transgenic tobacco lines overexpressing *PsAOS3* (OE-L1, OE-L2) before and after high-temperature stress (40 °C/35 °C 16 h light/8 h dark) for 96 h. (a) Antioxidant enzyme activities. (b) Jasmonic acid contents. (c) Photosynthetic characteristics. (d) Chlorophyll fluorescence parameters. The values represent the means ± SDs, and different letters indicate significant differences ($p < 0.05$).

4. Discussion

JA is a major stress-responsive hormone that coordinates plant growth, development, and defense under adverse conditions, including high-temperature [38] and drought [39]. Coordinated control of JA biosynthesis and signaling is thought to be necessary for the timely activation of cellular protective responses [40]. Altering the expression or activity of biosynthetic enzymes can modify JA production and thereby reshape downstream defense networks. In this study, we examined the function of *PsAOS3* during high-temperature stress in tree peony and investigated whether its effect on thermotolerance is mediated through JA accumulation.

AOS converts 13-HPOT into an unstable allene oxide in the JA-biosynthetic pathway. This first committed reaction constitutes an important control point for endogenous JA production [25]. AOS proteins belong to the CYP74 family and have been reported at the chloroplast inner membrane or PM; their catalytic function depends on conserved features that include the I-helix, ETLR motif, and heme-binding domain [41]. Sequence analysis showed that *PsAOS3* contains each of these characteristic regions and is highly homologous to AOS3 proteins from other species. Subcellular localization analysis showed that *PsAOS3* is localized to the plasma membrane. Although the early steps of JA biosynthesis are generally considered to occur in chloroplasts, AOS proteins are not invariably confined to this organelle; for example, the sugarcane *ShAOS1* was also reported to locate at the plasma membrane [42]. Thus, the plasma membrane localization of *PsAOS3* may reflect a species- or tissue-specific variation in AOS distribution.

The functions of AOS family members during high-temperature stress remain incompletely understood. Available evidence nevertheless suggests that high temperatures can rapidly activate genes in the JA-biosynthetic pathway and that these genes contribute positively to thermotolerance [43]. In cotton, *GhAOS* helps preserve circadian homeostasis and antioxidant capacity through JA biosynthesis, thereby maintaining male fertility and reducing high-temperature induced bud abscission and abortion [44]. In eggplant, *SmLOX4* and *SmLOX5* contribute to JA production and signaling, resulting in greater high-temperature tolerance and reduced injury [18]. Comparable functional evidence for AOS genes in woody ornamental species has not been reported. Here, exposure to 40 °C

increased *PsAOS3* transcript abundance by approximately 15-fold. This pronounced induction implicates *PsAOS3* as a high-temperature-responsive component that may connect temperature perception with enhanced JA production in tree peony.

To define the genetic contribution of *PsAOS3*, we suppressed its expression in tree peony using VIGS. *PsAOS3* silencing increased high-temperature sensitivity and produced broad physiological and metabolic changes. Under high-temperature stress, silenced plants showed lower SPAD values, higher b^* values, and more rapid leaf yellowing, consistent with a marked loss of chlorophyll. Similar senescence-associated phenotypes have been described in high-temperature-treated JA-deficient mutants [45]. JA content was approximately 40% lower in the silenced plants than in the control, supporting a role for *PsAOS3* in maintaining endogenous JA production under high temperatures. *PsAOS3*-silenced leaves also accumulated more H_2O_2 and $O_2^{\cdot-}$. Reduced JA availability may have weakened JA-dependent antioxidant defenses: SOD, POD, and CAT activities all declined, limiting ROS-scavenging capacity. This interpretation agrees with evidence that JA signaling promotes antioxidant enzyme expression and activity to preserve redox balance during high-temperature stress [46]. Consistent with increased oxidative injury, the silenced plants had higher MDA content and REC, indicating enhanced lipid peroxidation and membrane damage. They also displayed lower Fv/Fm and higher qN, suggesting impaired PSII photochemical performance and greater dissipation of excitation energy. Collectively, these changes indicate that loss of *PsAOS3* reduces JA accumulation and compromises enzymatic antioxidant protection, thereby intensifying oxidative, membrane, and photosynthetic damage under high-temperature stress [30,47]. Despite moderate silencing efficiency (37.6% and 44.1%), the consistent phenotypes of two independent lines suggest that the observed effects are not attributable to off-target silencing, although the efficiency may have constrained the phenotypic magnitude.

We next tested whether *PsAOS3* function is conserved across species by overexpressing it in tobacco. Transgenic plants were more high-temperature tolerant than wild-type (WT) plants and accumulated 2.1-fold more endogenous JA. They also maintained better ROS homeostasis, PSII performance, and membrane stability. Previous studies have shown that overexpression of JA-biosynthetic genes can enhance thermotolerance in model plants [48]. In our experiment, *PsAOS3*-overexpressing tobacco exhibited less leaf yellowing, lower ROS accumulation, and higher POD, SOD, and CAT activities than WT plants. These responses resemble those reported for eggplant overexpressing *SmLOX4* or *SmLOX5*, in which increased endogenous JA and antioxidant capacity improved high-temperature tolerance [18]. We therefore propose that *PsAOS3* supports JA production during high-temperature exposure, enabling sustained JA signaling and antioxidant defense. This response limits secondary oxidative injury and promotes an active thermotolerant state.

Compared with herbaceous crops, the molecular basis of JA-dependent thermotolerance in woody ornamentals has received limited attention [49]. Our results identify *PsAOS3* as a positive regulator of high-temperature tolerance in tree peony and support a pathway linking JA accumulation with antioxidant protection and maintenance of photosynthesis. By combining transcriptome-guided candidate selection with gene-silencing and overexpression analyses, this study shows that *PsAOS3* promotes endogenous JA accumulation, reinforces antioxidant defenses, and protects membranes and the photosynthetic apparatus during high-temperature stress. *PsAOS3* thus expands current understanding of JA-mediated high-temperature responses in woody plants and provides a candidate gene for further investigation. However, because the current data were obtained under controlled-growth chamber conditions, future field studies are needed to validate its role in thermotolerance. In addition, identifying upstream transcription factors that regulate *PsAOS3* expression would help elucidate the regulatory network underlying JA-dependent high-temperature adaptation.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agriculture16151678/s1>, Figure S1. Phylogenetic analysis of PsAOS3 with representative members of the CYP74A, CYP74B, CYP74C, and CYP74D subfamilies from other plant species [27]. Figure S2: Identification of the PsAOS3-silenced tree peony. (a) PCR identification of the PsAOS3-silenced plants. (b) RT-qPCR identification of the PsAOS3-silenced plants. The values represent the means $\pm$ SDs, and different letters indicate significant differences ($p < 0.05$); Figure S3: PCR identification of transgenic tobacco lines overexpressing PsAOS3; Table S1: Gene-specific primers used for RT-qPCR detection in this study.

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