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Ethylene Response Factor OfERF16 Delays Postharvest Petal Senescence and Alters Floral Architecture in Osmanthus fragrans

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Abstract

In *Osmanthus fragrans*, the short postharvest life of individual flowers limits ornamental display and fragrance-related utilization, whereas the transcriptional regulators underlying petal senescence remain insufficiently defined. To clarify the role of *OfERF16*, expression profiling was integrated with subcellular localization, transient overexpression in detached *O. fragrans*petals, stable overexpression in tobacco, physiological measurements, and interaction analyses. *OfERF16*transcripts accumulated strongly at the late flowering stage, and the protein localized to the nucleus and cell periphery. *OfERF16* overexpression delayed senescence in detached *O. fragrans*petals and in transgenic tobacco flowers. This response was accompanied by reduced expression of senescence-associated markers, lower malondialdehyde and H2O2 accumulation, and higher peroxidase activity and soluble protein content. In tobacco, *OfERF16* overexpression also altered floral architecture, particularly by promoting stamen elongation relative to the pistil. Yeast two-hybrid screening identified candidate interactors, and bimolecular fluorescence complementation confirmed an *in planta* interaction between OfERF16 and the ABA-related transcription factor OfMYB306. Yeast one-hybrid and dual-luciferase assays showed that OfbHLH68 associates with the *OfERF16* promoter and represses its transcriptional activity. These results place OfERF16 within a regulatory framework linking ethylene-responsive regulation, ABA-associated signaling, and redox homeostasis during postharvest petal aging, providing a mechanistic basis for improving floral longevity in *O. fragrans*.

Key Message

OfERF16 delays petal senescence by coordinating redox homeostasis, floral architecture, and ABA-associated regulation through interaction with OfMYB306 and transcriptional repression by OfbHLH68.

1. Introduction

Osmanthus fragrans is a widely cultivated ornamental tree in East Asia and is valued for both its floral fragrance and horticultural use. Its flowers are used for ornamental display as well as fragrance products, foods, beverages, and traditional medicinal preparations. Previous studies have also reported antioxidant and anti-inflammatory activities in *O. fragrans* flowers, highlighting their value as aromatic plant resources (Huang et al. 2023; Hung et al. 2012, 2013; Song et al. 2021). However, the flowering period of many cultivars is highly concentrated, and individual flowers senesce rapidly after harvest or detachment, often within less than one week (Wang et al. 2022). More importantly, the interval from anthesis to visible senescence is short, with the optimal harvest window generally restricted to 2–3 days (Xia et al. 2024). This narrow window constrains postharvest display quality, fragrance extraction, processing continuity, and practical utilization of the flowers (Lee et al. 2020; Usuki and Munakata, 2017). Extending postharvest floral longevity has therefore become an important goal for improving the use value of *O. fragrans* flowers (Han et al. 2022; Yang et al. 2025; Zhong et al. 2024).

Petal senescence is accompanied by coordinated cellular deterioration, physiological decline, and large-scale transcriptional reprogramming (Cao et al. 2024; Zou et al. 2024). Flower senescence and abscission are the most typical manifestations, with programmed cell death—particularly the regulatory mechanisms centered on hormone signaling pathways—playing a pivotal role in petal senescence (Ma et al. 2018; Mayak and Halevy, 1972; Wang et al. 2023). The progression of petal senescence is strongly influenced by the balance and interaction of endogenous hormones. Among these, ethylene and abscisic acid are recognized as typical senescence-promoting hormones, while cytokinins and gibberellins generally delay senescence. As a flower highly sensitive to ethylene, *O. fragrans* is significantly regulated by ethylene during senescence (Chen et al. 2020, 2024). Elevated endogenous ethylene levels are highly synchronized with senescence traits such as petal browning and abscission. However, the molecular regulatory network underlying petal senescence in *O. fragrans* remains unclear, and the relevant mechanisms warrant further in-depth investigation.

Members of the AP2/ERF family are plant-specific transcription factors with broad functions in development and stress-responsive regulation (Chen et al. 2022; Nakano et al. 2014). Among them, the ERF subfamily has been extensively reported to regulate floral organ senescence (Chung et al. 2016; Liu et al. 2011; Pré et al. 2008). In *Camellia*, genes associated with ethylene biosynthesis and signaling are upregulated during flower senescence, supporting a key role for ethylene in this process (Liu et al. 2022). In carnation, variation in ethylene biosynthesis is closely associated with differences in senescence progression (Brandt and Woodson, 1992). In rose, silencing two ERF transcription factors (*RhERF1* and *RhERF4*) regulated by ethylene and auxin reduces pectin content in abscission-zone tissues, revealing their function in petal abscission (Gao et al. 2019). Furthermore, our previous systematic analysis of the ERF transcription factor family in *O. fragrans* identified several candidate genes potentially involved in petal senescence regulation (Chen et al. 2024). Among these candidates, *OfERF16* showed pronounced late-stage induction during flower development, making it a strong candidate regulator of petal senescence. Nonetheless, the biological function of *OfERF16*, its position within hormone-related regulatory networks, and its specific contribution to floral developmental traits remain unclear.

In this study, expression analysis, localization assays, transient and stable overexpression, physiological measurements, and interaction analyses were combined to investigate the role of *OfERF16* in petal senescence and floral development. Particular attention was given to detached petals and post-anthesis flower longevity because these traits directly affect postharvest quality maintenance in *O. fragrans*. It was specifically asked whether *OfERF16* attenuates senescence-associated deterioration, whether it interacts with ABA-related regulators, and whether it is subject to upstream transcriptional repression. These analyses were designed to define how *OfERF16* is positioned within a regulatory framework associated with ethylene response, ABA-related signaling, and redox balance during postharvest floral senescence.

2. Materials and Methods

2.1 Plant material and sample collection

Eight- to ten-year-old *O. fragrans* 'Rixiang Gui' plants cultivated at Nanjing Forestry University were used in this study. Petal samples were collected at five developmental stages: S1, bud-eye stage; S2, Linggeng stage (bud enlargement stage); S3, initial flowering stage; S4, full flowering stage; and S5, late flowering stage. For each stage, three independent biological replicates were collected from different plants. Fresh petals were immediately frozen in liquid nitrogen and stored at −80°C until use.

2.2 RNA extraction and qRT-PCR analysis

Total RNA was isolated from frozen petal samples using TRIzol reagent (Tiangen, Beijing, China). Genomic DNA was removed and first-strand cDNA was synthesized using EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Specific primers (Table S1) were designed using Primer Premier 5.0 and validated for amplification specificity and efficiency. qRT-PCR was performed on a QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA) using TB Green Premix Ex Taq II (Tli RNaseH Plus; Takara, Japan). Each 10 μL reaction contained 5 μL premix, 0.4 μL of each primer (10 μM), 0.2 μL ROX Reference Dye II, 1 μL cDNA, and 3 μL ddH_2O. The amplification program consisted of 95°C for 3 min, followed by 40 cycles of 95°C for 45 s, 60°C for 30 s, and 72°C for 30 s. *OfRAN* (F: AGAACCGACAGGTGAAGGCAA; R: TGGCAAGGTACAGAAAGGGCT) was used as the internal reference gene for normalization. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Three biological replicates and three technical replicates were analyzed for each sample.

2.3 Gene cloning and vector construction

The coding sequence of *OfERF16* was amplified from S4 stage *O. fragrans* cDNA using primers listed in Table S1. PCR products were separated on 1.2% agarose gels, purified with the Universal DNA Purification Kit (Tiangen, Beijing, China), and ligated into the pSuper1300 vector after *BamHI*/*SpeI* digestion. Recombinant plasmids were transformed into *Escherichia coli* DH5α, and positive colonies were confirmed by colony PCR and Sanger sequencing (Tsingke Biotech, Nanjing, China).

2.4 Bioinformatics analysis

The *OfERF16* coding sequence was queried against the NCBI non-redundant protein database using BLASTp. Homologous proteins with high sequence similarity were aligned using MUSCLE in MEGA 11, and a neighbor-joining phylogenetic tree was constructed with 1,000 bootstrap replicates. Conserved domains were analyzed using the NCBI Conserved Domain Database and InterProScan. Predicted protein features, including secondary structure and transmembrane helices, were analyzed using PSIPRED and TMHMM. The upstream genomic region of *OfERF16* was further analyzed for putative cis-elements using PlantCARE.

2.5 Subcellular localization of OfERF16

For subcellular localization, the *OfERF16* coding sequence was fused in frame with GFP in the pSuper1300 vector. The resulting construct and the empty vector were introduced into *Agrobacterium tumefaciens* GV3101. Agrobacterial cultures were resuspended in infiltration buffer (10 mM $MgCl_2$, 120 μM acetosyringone, pH 5.6) to an OD_{600} of 0.6 and incubated for 2 h at room temperature. The

suspensions were infiltrated into *N. benthamiana* leaves together with a nuclear marker. GFP and marker fluorescence were observed 48–72 h after infiltration using a Zeiss LSM 880 confocal microscope.

2.6 Transient expression in *O. fragrans* petals

For homologous transient assays, the pSuper1300-*OfERF16* construct or the empty vector was introduced into *A. tumefaciens* GV3101. Bacterial suspensions were prepared in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.6, 150 μM acetosyringone) to an OD_{600} of 0.6–0.8 and incubated for 2 h at room temperature. Fresh S4-stage *O. fragrans* petals were vacuum-infiltrated at −0.075 MPa for 15 min, followed by a second infiltration cycle. Petals were placed on 0.5% agar plates and incubated in the dark at 25°C for 48 h before subsequent analyses.

2.7 Stable transformation and phenotypic analysis in tobacco

The pSuper1300-*OfERF16* construct was introduced into *A. tumefaciens* GV3101 and used for leaf-disc transformation of *N. tabacum*. Leaf discs from 4- to 6-week-old plants were co-cultivated on MS medium supplemented with 1 mg/L 6-BA and 0.1 mg/L NAA, then transferred to selection medium containing kanamycin and cefotaxime. Resistant shoots were rooted on kanamycin-containing medium, and positive transformants were verified by PCR. Wild-type plants were used as controls for phenotypic, molecular, and physiological analyses.

2.8 Physiological measurements

All physiological measurements were performed using fresh tissues with at least three biological replicates. POD activity was determined colorimetrically from phosphate-buffer extracts. H_2O_2 content was measured using the titanium sulfate method. MDA content was determined using the thiobarbituric acid method, and soluble protein content was measured using the Bradford assay. Physiological indices were calculated according to the corresponding assay procedures using standard curves or extinction coefficients where applicable.

2.9 Yeast two-hybrid (Y2H) screening and validation

The *OfERF16* coding sequence was cloned into pGBKT7 and used as bait for Y2H assays in *Saccharomyces cerevisiae* Y2HGold. After testing for bait autoactivation, the screen was conducted against a floral cDNA library under selective conditions containing 500 ng/mL AbA. Positive clones were recovered, sequenced, and re-tested by directed Y2H assays using pGADT7-derived prey constructs.

2.10 Bimolecular fluorescence complementation

Candidate interaction partners were cloned into pSPYNE and pSPYCE vectors and introduced into *A. tumefaciens* GV3101. Agrobacterial suspensions were co-infiltrated into *N. benthamiana* leaves. YFP fluorescence was examined 48–72 h later using confocal microscopy to verify protein-protein interactions in plant cells.

2.11 Yeast one-hybrid (Y1H) screening and validation

The *OfERF16* promoter region was cloned into pHIS2 and used as bait in *Saccharomyces cerevisiae* Y187. Optimal suppression of bait autoactivation was established using 3-amino-1,2,4-triazole (3-AT), and a floral cDNA library was screened under selective conditions containing 50 mM 3-AT. Candidate transcription factors were recovered, sequenced, and verified by directed Y1H assays.

2.12 Dual-luciferase reporter assay

The *OfERF16* promoter was inserted upstream of the LUC reporter in pGreenII 0800-LUC, and candidate transcription factors were cloned into pGreenII 62-SK. Reporter and effector constructs were co-infiltrated into *N. benthamiana* leaves. Luciferase activity was measured 72 h after infiltration using a Dual-Luciferase Reporter Assay System (Promega), and LUC/REN ratios from at least three biological replicates were used to quantify promoter activity.

2.13 Statistical analysis

All experiments included at least three independent biological replicates. Data are presented as mean ± SD. Statistical analyses were performed using GraphPad Prism 9.0. Comparisons between two groups were evaluated using Student's *t*-test, and multi-group comparisons were analyzed by one-way ANOVA as appropriate. Multiple comparisons after one-way ANOVA were performed using Tukey's test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1 *OfERF16* is induced during late flower development

qRT-PCR analysis across five floral stages revealed that *OfERF16* transcript abundance remained relatively low from S1 to S4 but increased sharply at S5 (Fig. 1A). This expression pattern was consistent with our previous transcriptome dataset, indicating that *OfERF16* is associated with the late phase of petal development. The sharp increase of *OfERF16* transcript abundance at S5 links this gene to the late phase of petal development and the onset of senescence-associated changes.

3.2 Phylogenetic analysis and sequence features of OfERF16

The AP2/ERF superfamily in *O. fragrans* contains 298 members, and the candidate analyzed here was designated *OfERF16* according to the nomenclature used in our previous dataset (Table S2). Phylogenetic analysis using homologous sequences from *Olea europaea var. sylvestris*, *Abeliophyllum distichum*, *Forsythia ovata*, *Rehmannia glutinosa*, and *Sesamum alatum* positioned *OfERF16* closest to the olive homolog (Fig. 1B), consistent with the evolutionary proximity of Oleaceae species.

Multiple-sequence alignment further showed that *OfERF16* contains the conserved AP2/ERF DNA-binding domain (Fig. 1C). The phylogenetic position and conserved AP2/ERF domain support the assignment of *OfERF16* to the ERF family and are consistent with a role in transcriptional regulation during flower aging.

3.3 Subcellular localization of OfERF16

Transient expression of *OfERF16*-GFP in *N. benthamiana* leaves revealed fluorescence in the nucleus and at the cell periphery (Fig. 2). By contrast, free GFP from the empty vector was distributed broadly throughout the cell. This localization pattern is consistent with a role in nuclear transcriptional regulation and suggests a possible association with peripheral cellular compartments.

3.4 Transient overexpression of *OfERF16* suppresses senescence-associated changes in *O. fragrans* petals

To assess *OfERF16* function in its native species, we transiently overexpressed *OfERF16* in S4 petals of *O. fragrans*. After 48 h, *OfERF16* transcript abundance in infiltrated petals was approximately threefold higher than that in the empty-vector control (Fig. 3B), confirming successful transient expression. Visible differences between treatments were modest over this short assay window (Fig. 3A). Nevertheless, *OfERF16*-overexpressing petals exhibited lower expression of *OfSAG21* and *OfACO3*, two markers associated with senescence progression and ethylene biosynthesis (Fig. 3B). The physiological measurements followed the same trend: POD activity and soluble protein content increased, whereas MDA and H_2O_2 levels decreased significantly in *OfERF16*-overexpressing petals (Fig. 3C). Although visible morphological divergence remained limited within 48 h, the molecular and physiological data consistently pointed to delayed senescence in *OfERF16*-overexpressing petals.

3.5 *OfERF16* overexpression delays floral senescence and alters floral architecture in tobacco

To test whether *OfERF16* affects flower development and senescence, stable overexpression lines were generated in *N. tabacum* (Fig. 4A). *OfERF16* transcript abundance was highest at the late flowering stages in tobacco petals (S4–S5), showing a late-stage accumulation pattern broadly consistent with that observed in *O. fragrans* (Fig. 4B). Compared with wild-type (WT) plants, the overexpression (OE) lines flowered earlier at the whole-plant level, whereas the lifespan of individual flowers from opening to abscission was extended by approximately 2 d (Fig. 4C). In addition, more than 75% of transgenic flowers showed elongated stamens that exceeded the pistil, resulting in a marked change in floral architecture (Fig. 4D). These observations suggest that *OfERF16* affects whole-plant flowering behavior and post-anthesis senescence through at least partly distinct processes.

At the molecular level, overexpression of *OfERF16* attenuated the expression of senescence-associated genes. *NtSAG12* transcript abundance was markedly higher in WT petals than in OE petals at S4, and this

difference became smaller by S5; *NtACO1* showed the same overall tendency, with higher expression in WT during the active senescence phase (Fig. 5A). Physiological assays further showed that MDA and H_2O_2 contents were 35–40% lower in OE petals than in WT petals (Fig. 5B), indicating reduced oxidative damage. Taken together, the tobacco data support a role for *OfERF16* in delaying senescence-associated deterioration while also influencing floral organ development.

3.6 Y2H screening identifies candidate OfERF16-interacting proteins

To identify proteins associated with *OfERF16*, we used *OfERF16* as bait in a Y2H screen against a floral cDNA library prepared from *O. fragrans* flowers. Because the bait showed autoactivation, aureobasidin A (AbA) was titrated to establish stringent screening conditions. Growth of the bait strain was fully suppressed on selective medium containing 500 ng mL^{-1} AbA, and this concentration was therefore used for subsequent screening (Fig. S1). Library screening yielded 52 positive clones (Fig. S2). After sequencing and removal of redundant hits, 33 unique candidate proteins were retained. On the basis of annotation and their possible relevance to senescence or hormone signaling, four candidates, namely OfRAP2-2, OfbHLH094, OfMYB306, and OfSAMS2, were selected for directed validation.

3.7 *OfMYB306* is a robust *in planta* interaction partner of OfERF16

Directed Y2H assays showed that *OfERF16* supported yeast growth with OfRAP2-2, OfbHLH094, and *OfMYB306* on stringent selective medium, whereas no interaction was detected with OfSAMS2 (Fig. 6). These assays identified a small set of candidate interactors but did not distinguish which associations were maintained in plant cells. We therefore carried out bimolecular fluorescence complementation (BiFC) assays in *N. benthamiana* leaves. Among the combinations tested, only YCE-*OfERF16* and YNE-*OfMYB306* produced a reproducible YFP signal, which was detected in the nucleus and at the cell periphery (Fig. 7). No fluorescence was observed in the corresponding negative controls or in the other candidate combinations. Thus, *OfMYB306* was the only candidate interaction partner confirmed both in yeast and *in planta*, highlighting it as the most robust component of the *OfERF16* protein interaction network identified here.

3.8 Yeast one-hybrid screening identifies a candidate upstream regulator of OfERF16

To identify upstream regulators of *OfERF16*, we screened a floral cDNA library using the *OfERF16* promoter as bait in a yeast one-hybrid (Y1H) system. Background activation of the bait construct was suppressed by 50 mM 3-AT, which was therefore used for the library screen (Fig. S3). The screen produced 49 positive clones (Fig. S4), corresponding to 34 non-redundant candidate transcription factors. Among these, *OfbHLH68* was prioritized for further study because of its predicted involvement in stress-responsive transcription.

3.9 *OfbHLH68* binds the *OfERF16* promoter in yeast and represses its transcriptional activity

Directed Y1H assays confirmed that *OfbHLH68* binds the *OfERF16* promoter in yeast: cotransformed strains grew robustly on selective medium containing 50 mM 3-AT, whereas the negative control did not (Fig. 8). To determine the functional consequence of this association, we performed dual-luciferase assays in *N. benthamiana* leaves. Co-expression of *OfbHLH68* with the *OfERF16* promoter reporter reduced LUC/REN activity by approximately 65% relative to the empty-vector control (Fig. 9), indicating that *OfbHLH68* acts as a transcriptional repressor of *OfERF16*. These assays support the point that *OfbHLH68* acts upstream of *OfERF16* and represses its transcriptional activity.

3.10 A working model summarizes the role of *OfERF16* in petal senescence and floral architecture

Based on the experimental evidence obtained in this study, a working model was proposed to summarize the role of *OfERF16* in petal senescence and floral architecture (Fig. 10). *OfERF16* was strongly induced at the late stage of flower development and localized predominantly to the nucleus and cell periphery. Functional analyses in both *O. fragrans* petals and transgenic tobacco showed that elevated *OfERF16* expression suppressed the expression of senescence-associated genes, reduced H_2O_2 and MDA accumulation, and maintained higher antioxidant activity and soluble protein content, thereby delaying petal senescence. At the regulatory level, *OfbHLH68* bound the *OfERF16* promoter in yeast and repressed its transcriptional activity, whereas *OfERF16* interacted with *OfMYB306* in plant cells, suggesting a possible connection between ethylene-responsive regulation and ABA-associated signaling. In addition to delaying petal senescence, *OfERF16* overexpression also altered floral architecture in tobacco, particularly by promoting stamen elongation relative to the pistil. Overall, the data support a model in which *OfERF16* attenuates petal senescence while also contributing to the coordination of floral longevity and floral organ development.

4. Discussion

A central finding of this study is that *OfERF16* accumulates during the late floral stage and functions as a senescence-attenuating regulator in both transient and stable overexpression systems (Ma et al. 2024; Nie and Wang, 2023; Su et al. 2025). For example, genome-wide identification of the AP2/ERF gene family has been performed in *Pinus massoniana*, *Morus notabilis*, and *Prunus avium*, laying a foundation for subsequent functional studies (Dou et al. 2024; Sun et al. 2022; Zhang et al. 2026). *OfERF16* was selected from our previous genome-wide analysis of the *O. fragrans* ERF family because of its strong late-stage induction pattern. Expression analysis revealed that *OfERF16* transcript levels were significantly higher at the S5 stage than at the other four stages, suggesting that this gene may play an important role in petal senescence of *O. fragrans* (Fig. 1A). Gene expression products typically need to be localized to specific subcellular compartments to exert their biological functions and thereby regulate

plant physiological activities (Wang et al. 2018; Yu et al. 2022). The observed localization of OfERF16 to the nucleus and cell periphery is compatible with a transcriptional role accompanied by possible peripheral signaling association (Fig. 2). Nuclear localization is consistent with its role as a transcriptional regulator, whereas the peripheral signal suggests a potential association with membrane-proximal signaling contexts. Whether this peripheral signal reflects a stable membrane-associated pool, transient trafficking processes, or dynamic redistribution during senescence remains to be clarified in future studies.

In general, functional validation of genes relies on efficient and stable genetic transformation systems. However, establishing stable transformation systems for woody plants remains challenging (Maharjan et al. 2025). Plant transient transformation systems offer a feasible alternative strategy for gene functional validation. Following transient overexpression of *OfERF16* in *O. fragrans* petals, its expression level was significantly higher than that in the empty vector control, indicating successful transfer of *OfERF16* into *O. fragrans* petals (Fig. 3B). In plants, changes in the expression of SAG and ACO genes are frequently used as markers of flower senescence; decreased expression of these two genes typically indirectly reflects a delay in the senescence process (Lü et al. 2014; Trivellini et al. 2016). In this study, upon overexpression of *OfERF16*, the expression levels of SAG and ACO in *O. fragrans* petals were significantly reduced, suggesting that *OfERF16* may function to delay petal senescence (Fig. 3B). Although ethylene production was not directly measured, the coordinated downregulation of these marker genes strongly indicates that *OfERF16* suppresses core components of the senescence program. Reactive oxygen species are important signaling molecules during plant senescence, and their excessive accumulation can damage cell membranes and exacerbate senescence-related injury (Salleh et al. 2016; Tewari et al. 2009). In this study, the contents of H_2O_2 and MDA were significantly lower in *OfERF16*-overexpressing *O. fragrans* petals than in the empty vector control, indicating that petal senescence was alleviated (Fig. 3C). These results further support the notion that *OfERF16* may function to delay petal senescence in *O. fragrans*. Additionally, we performed heterologous stable transformation of *OfERF16* in the model plant *Nicotiana tabacum*. Petal senescence was significantly delayed in transgenic tobacco lines, accompanied by markedly reduced expression levels of SAG and ACO (Figs. 4C and 5A). Moreover, decreased H_2O_2 and MDA levels also indicated reduced oxidative damage in petals (Fig. 5B). These data suggest that *OfERF16* helps maintain petal longevity by attenuating senescence-associated transcription and limiting oxidative deterioration. The concordant trends observed in *O. fragrans* petals and tobacco indicate that the senescence-related function of *OfERF16* may be at least partly conserved across species.

The interaction and promoter-association assays further narrowed the regulatory context in which OfERF16 operates (Kamigaki et al. 2016; Salinas et al. 2024; Sun et al. 2024). Among the proteins tested, OfMYB306 was the only interactor supported by both yeast assays and BiFC in plant cells, making it the strongest interaction candidate identified in the present study. Given that MYB factors can participate in ABA-related regulation, the *OfERF16–OfMYB306* complex may represent a potential crosstalk node between ethylene-responsive transcription and ABA-associated signaling during petal senescence in *O. fragrans* (Fig. 7) (Lu et al. 2024). At the upstream regulatory level, *OfbHLH68* was shown to bind the

OfERF16 promoter in yeast and repress reporter gene expression (Figs. 8 and 9) (Cao et al. 2023; Wang et al. 2018). This result indicates that OfERF16 is not only associated with downstream senescence-related outputs but is also subject to upstream transcriptional repression. Collectively, the current evidence supports a model in which OfbHLH68 represses OfERF16 transcription, whereas OfERF16 may function together with OfMYB306 in pathways associated with senescence and floral organ maintenance (Figs. 8–10). Nevertheless, the precise biochemical consequences of the interaction between *OfERF16* and *OfMYB306* remain to be further elucidated.

In tobacco, overexpression of *OfERF16* accelerated the overall flowering time of whole plants but extended the longevity of individual flowers and significantly altered the stamen-to-pistil ratio (Fig. 4D). These phenotypes suggest that the function of *OfERF16* is not limited to senescence regulation but also affects broader patterns of floral development. These structural alterations suggest that regulators of senescence may also influence pathways controlling floral organ growth and proportion. Several limitations of this study should be acknowledged. First, the experimental evidence in *O. fragrans* is primarily based on transient overexpression, lacking validation from stable genetic transformation or gene knockout materials. Second, hormone contents were not directly quantified; therefore, conclusions regarding the involvement of ethylene and ABA remain indirect and have not been unequivocally confirmed at the biochemical level. Third, only one interacting partner of *OfERF16* has been identified in plant cells to date, and the direct downstream target genes of *OfERF16* remain unknown. Hence, future research should aim to establish stable genetic transformation systems in *O. fragrans*, quantify dynamic hormone changes, identify direct transcriptional targets of OfERF16, and dissect the functional mechanism of the OfbHLH68-OfERF16-OfMYB306 module in petal senescence at a more refined mechanistic level.

5. Conclusion

The present study identifies OfERF16 as a senescence-attenuating regulator in *O. fragrans*. Functional analyses in both *O. fragrans* petals and transgenic tobacco showed that OfERF16 suppresses senescence-associated gene expression and alleviates oxidative deterioration. The data further suggest that OfERF16 interacts in planta with OfMYB306 and is transcriptionally repressed by OfbHLH68, placing it within a regulatory framework associated with ethylene-responsive signaling, ABA-related regulation, and redox homeostasis. In addition to delaying petal senescence, OfERF16 also influences floral architecture, indicating that it contributes to the coordination of longevity and flower development. These findings provide a basis for future mechanistic studies and for strategies aimed at extending floral display life in *O. fragrans* and related ornamental species.

Declarations

Author Contribution Statement

G.C. conceived and designed the research, developed the methodology, conducted experiments, and wrote the original draft. Z.C. conducted experiments, curated data, and reviewed and edited the manuscript. F.C. conducted and validated experiments and reviewed and edited the manuscript. X.Q. conducted and validated experiments. H.C. conducted experiments and provided resources. H.G. conducted and validated experiments and reviewed and edited the manuscript. Y.Y. contributed methodology and formal analysis. L.W. supervised the work and provided resources. X.Y. supervised the work and reviewed and edited the manuscript. G.L. conceived and supervised the research, administered the project, and acquired funding. All authors read and approved the final manuscript.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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Author Contribution

G.C. conceived and designed the research, developed the methodology, conducted experiments, and wrote the original draft. Z.C. conducted experiments, curated data, and reviewed and edited the manuscript. F.C. conducted and validated experiments and reviewed and edited the manuscript. X.Q. conducted and validated experiments. H.C. conducted experiments and provided resources. H.G. conducted and validated experiments and reviewed and edited the manuscript. Y.Y. contributed methodology and formal analysis. L.W. supervised the work and provided resources. X.Y. supervised the work and reviewed and edited the manuscript. G.L. conceived and supervised the research, administered the project, and acquired funding. All authors read and approved the final manuscript.

Data Availability

All data generated or analyzed during this study are included in this article and its Supplementary Information.

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Figures

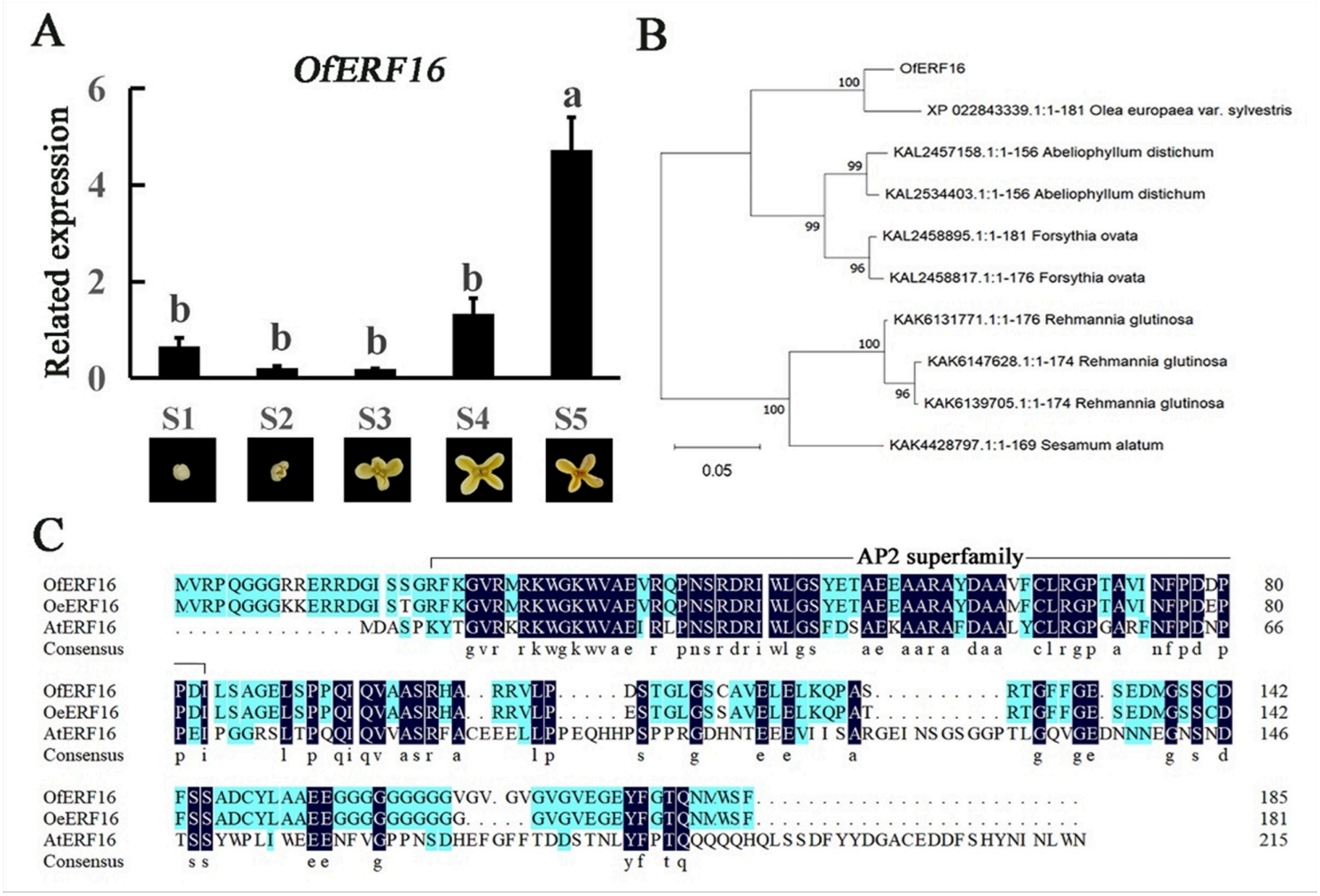


Figure 1

Expression and phylogenetic analysis of *OfERF16*. (A) Relative expression of *OfERF16* during five flowering stages of *O. fragrans*. Representative floral phenotypes at S1–S5: S1, bud-eye stage; S2, Linggeng stage (bud enlargement stage); S3, initial flowering stage; S4, full flowering stage; and S5, late flowering stage. (B) Phylogenetic tree of ERF16 homologs. (C) Multiple-sequence alignment of *OfERF16*, *AtERF16*, and *OeERF16*.

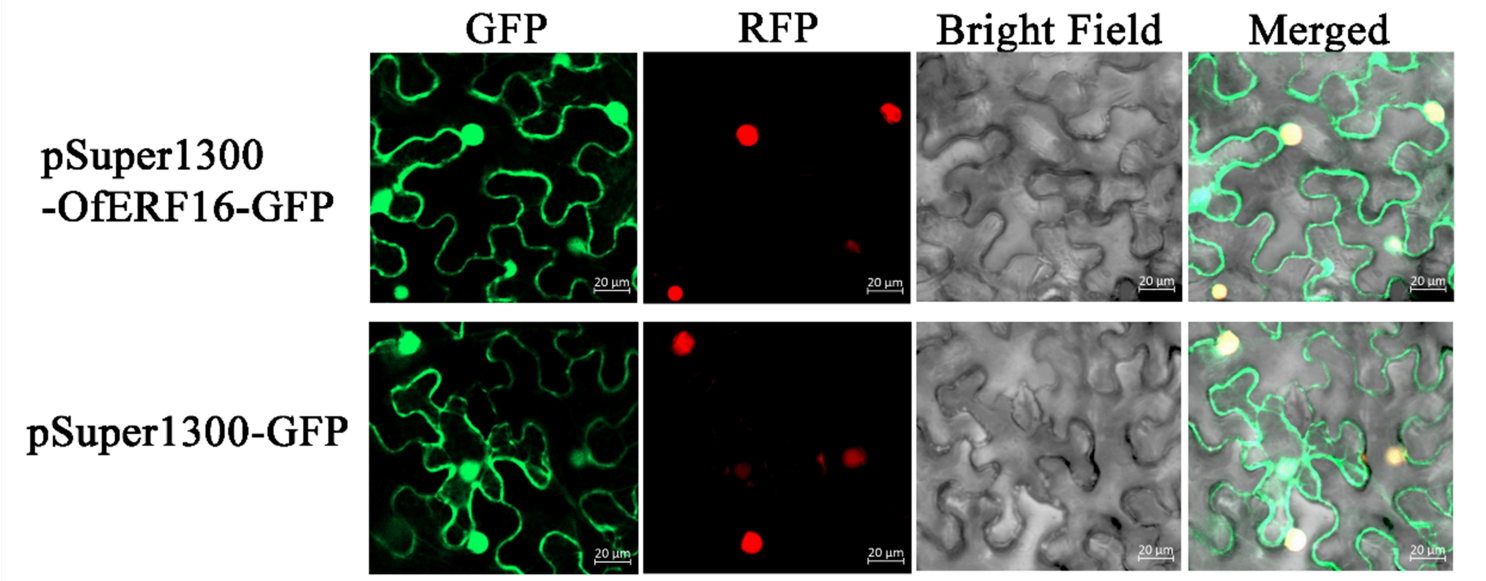


Figure 2

Subcellular localization of OfERF16-GFP in *N. benthamiana* leaves. GFP, green fluorescence; RFP, red fluorescence; Bright, bright field; Merge, merged image. Bar = 20 μm.

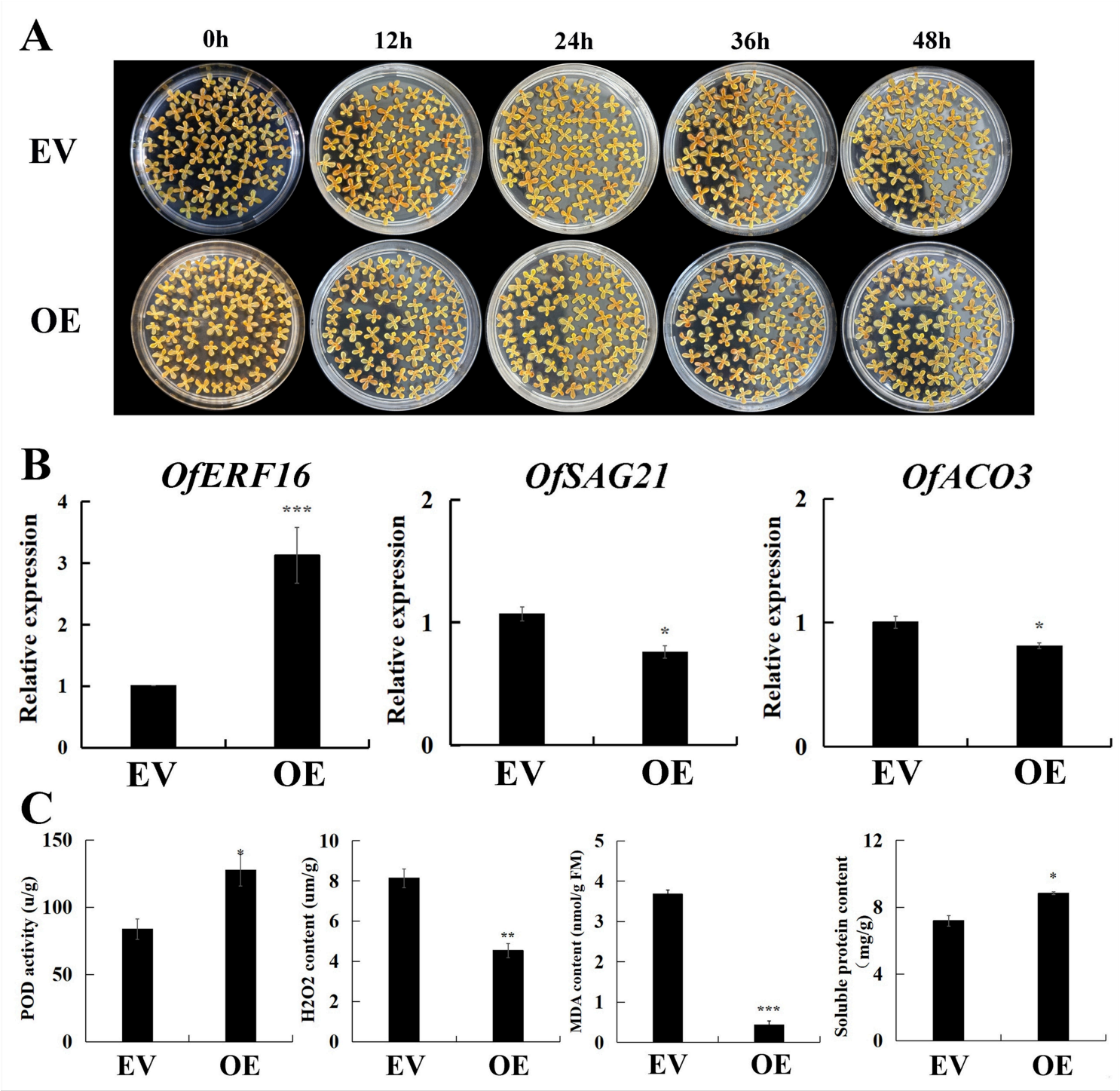


Figure 3

Functional analysis of *OfERF16* overexpression in *O. fragrans*petals. (A) Phenotypes of transiently transformed *O. fragrans* petals. EV, empty vector; OE, *OfERF16* overexpression. (B) Relative expression of OfERF16, *OfSAG21*, and *OfACO3* in transiently transformed *O. fragrans* petals. (C) Physiological indices in transiently transformed *O. fragrans*petals.

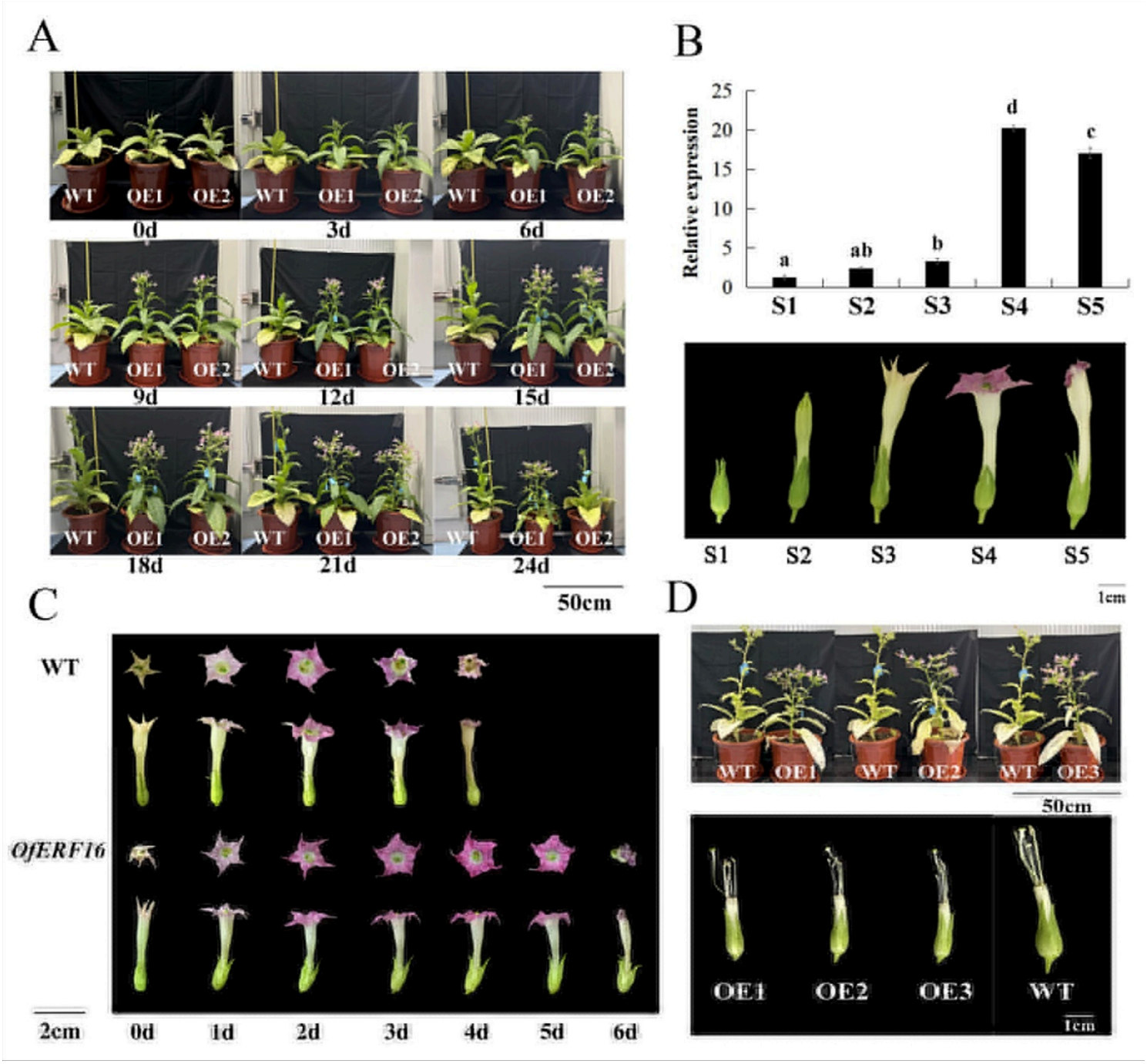


Figure 4

Functional analysis of *OfERF16* overexpression in tobacco petals. (A) Whole-plant flowering time of WT and OE lines. (B) Relative expression of *OfERF16* across the five flowering stages of *N. tabacum*. (C) Longevity of individual flowers from opening to abscission. (D) Representative floral phenotypes showing altered floral architecture in OE plants.

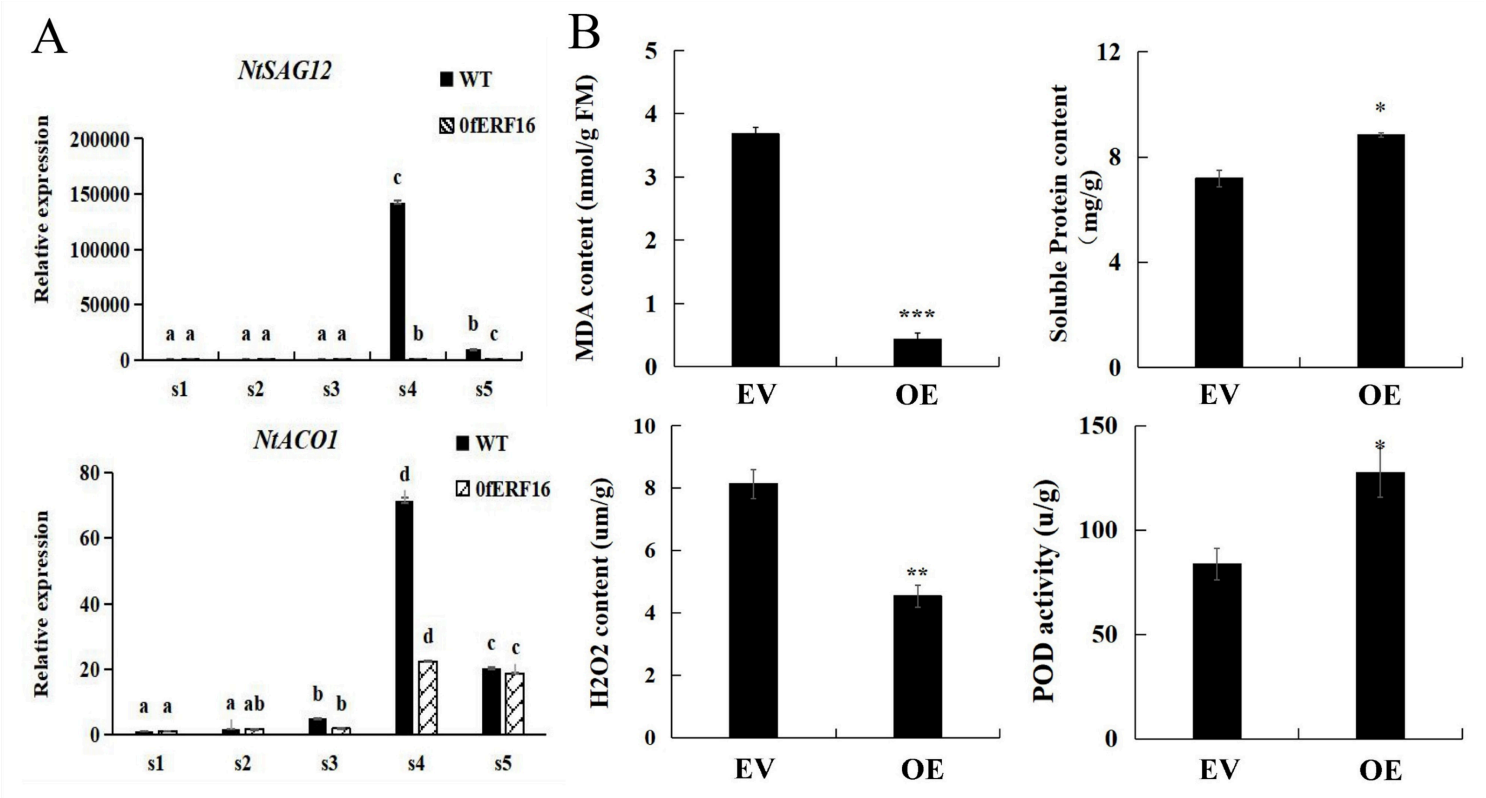


Figure 5

Effects of *OfERF16* overexpression on senescence-associated gene expression and physiological indices in tobacco petals. (A) Relative expression of *NtSAG12*and *NtACO1* in petals of WT and *OfERF16*-overexpressing lines at the indicated flowering stages. (B) MDA and H_2O_2 contents in WT and *OfERF16*-overexpressing petals.

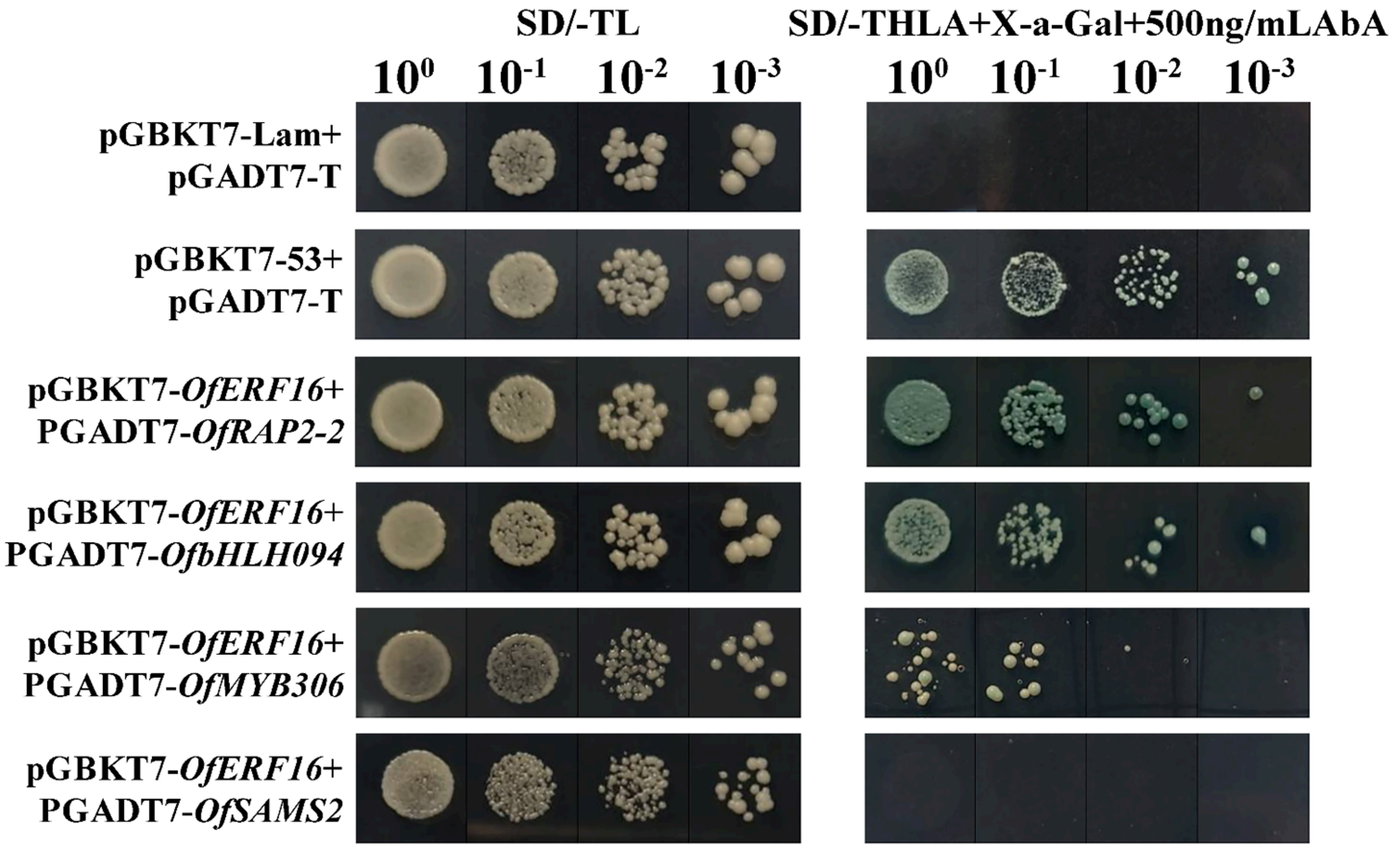


Figure 6

Directed yeast two-hybrid validation of candidate OfERF16-interacting proteins. Negative control, pGBKT7-Lam + pGADT7-T; positive control, pGBKT7-53 + pGADT7-T.

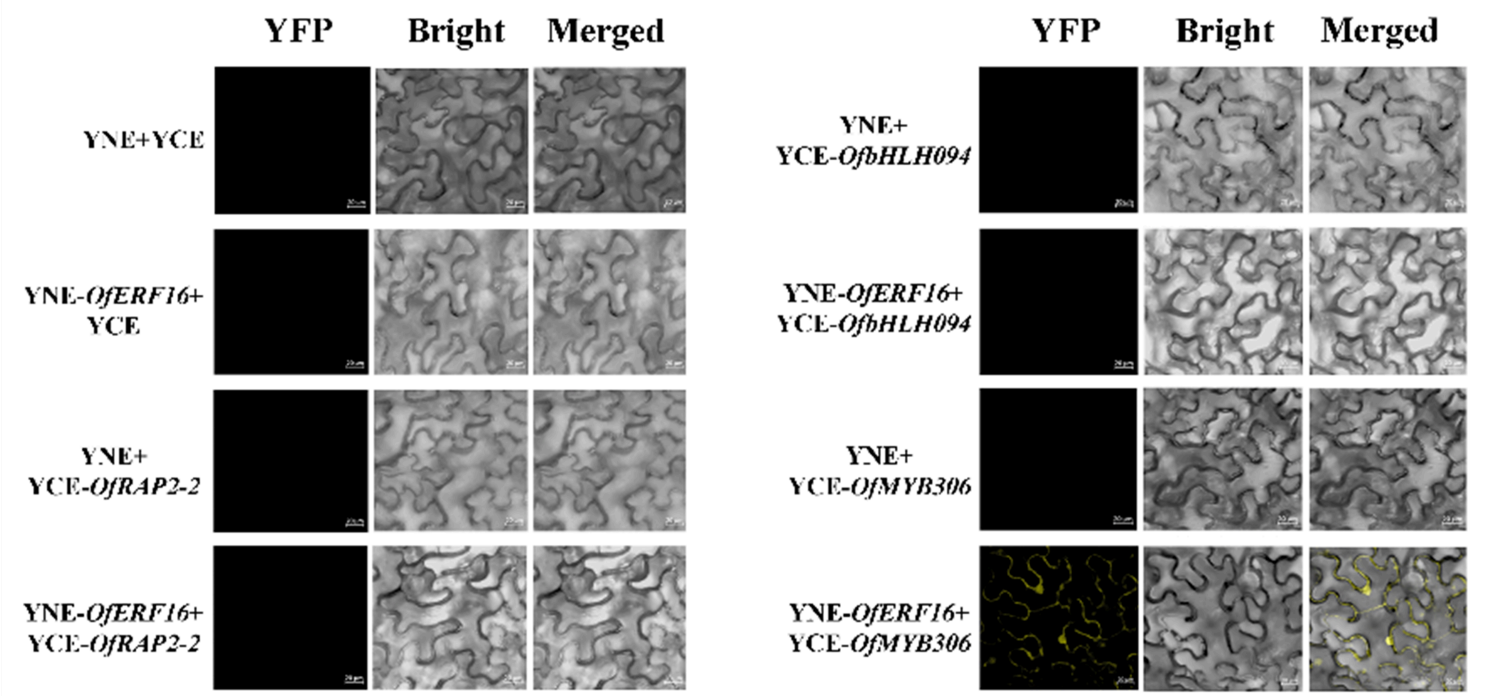


Figure 7

Bimolecular fluorescence complementation assay of *OfERF16* and candidate interaction partners in *N. benthamiana*leaves. YFP, yellow fluorescence; Bright, bright field; Merge, merged image.

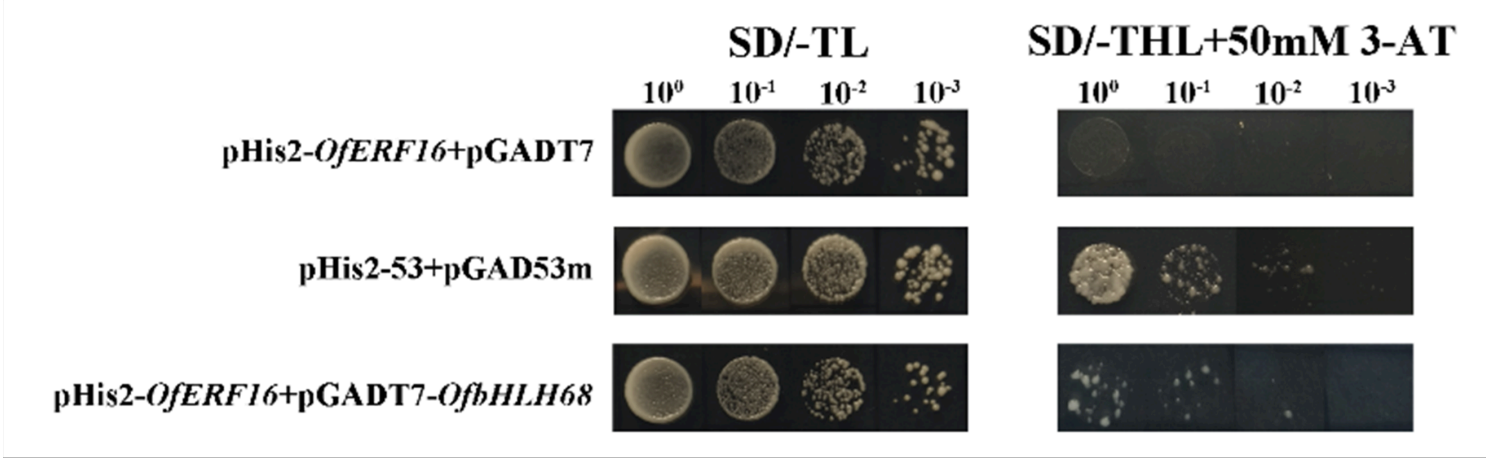


Figure 8

Directed yeast one-hybrid validation of the interaction between OfbHLH68 and the *OfERF16* promoter. Negative control, pHis2-*OfERF16* + pGADT7; positive control, pHis2-53 + pGAD53m.

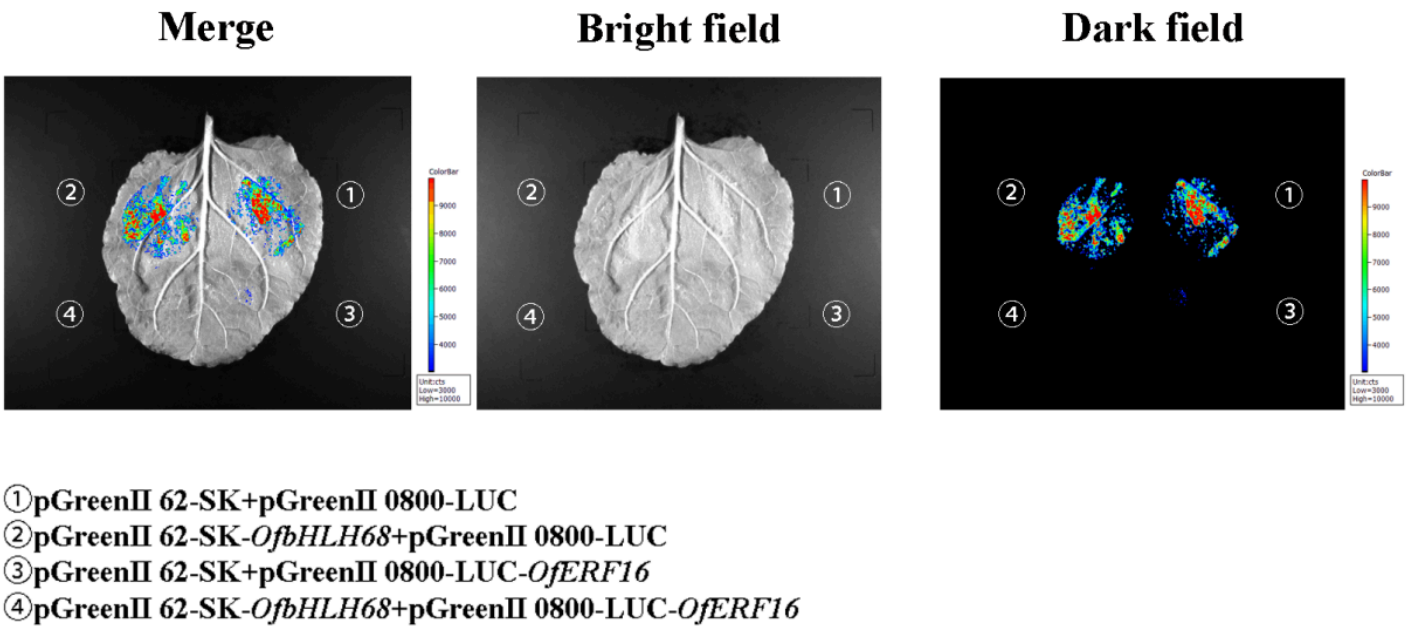


Figure 9

Dual-luciferase assay showing repression of the *OfERF16* promoter by OfbHLH68 in *N. benthamiana* leaves.

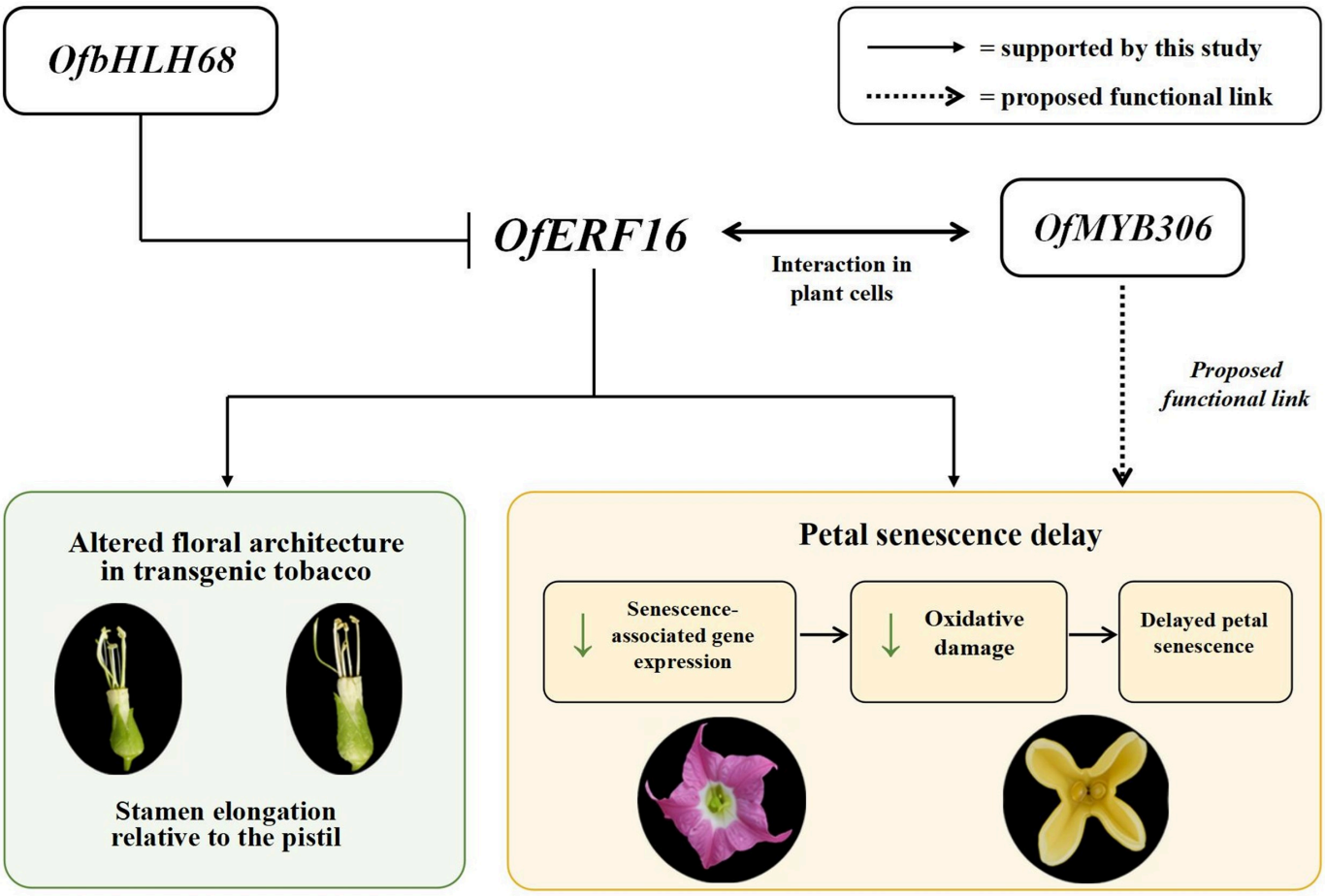


Figure 10

Proposed working model for the role of *OfERF16* in petal senescence and floral architecture. *OfbHLH68*represses *OfERF16* transcription, whereas *OfERF16* interacts with *OfMYB306* in plant cells. Increased *OfERF16* expression suppresses senescence-associated gene expression, alleviates oxidative damage, and delays petal senescence. In transgenic tobacco, *OfERF16*overexpression also alters floral architecture, particularly by promoting stamen elongation relative to the pistil. Solid lines indicate interactions supported by the present study, and dashed lines indicate proposed functional links requiring further validation.

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