The AP2/ERF Transcription Factor SlERF.F5 Functions in Leaf Senescence in Tomato

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Abstract

The process of plant senescence is complex and highly coordinated, and is regulated by many endogenous and environmental signals. Ethylene and jasmonic acid are well-known senescence inducers, but their molecular mechanisms for inducing leaf senescence have not been fully elucidated. Here, we studied a receptor gene downstream of an ethylene signal transduction pathway, ETHYLENE RESPONSE FACTOR F5 (*SlERF.F5*). The silence of *SlERF.F5* causes accelerated senescence induced by age, darkness, ethylene, and jasmonic acid. However, overexpression of *SlERF.F5* may delay leaf senescence. We further found that silencing of *SlERF.F5* inhibited the expression of chlorophyll-related genes *CHLH*, *CHLM*, *POR*, *CAO1*, *GUN4*, *PPH*, *SGR1*, *RBCS*, and *AUREA* genes, and the light-responsive *RBCS* and *LHCA1* gene. Moreover, silencing of *SlERF.F5* increases the sensitivity of *SlERF.F5*-RNAi lines to ethylene and jasmonic acid compared to wild type. In the dark-induced aging experiment, the qRT-PCR analysis showed the expression levels of genes related to the ethylene biosynthesis pathway and the jasmonic acid signaling pathway in *SlERF.F5*-RNAi lines increased compared with wild type. Yeast two-hybrid experiments showed that SlERF.F5 and SlMYC2 (a transcription factor downstream of the JA receptor) can interact physically, thereby mediating the role of *SlERF.F5* in jasmonic acid-induced leaf senescence. Collectively, our research provides new insights into how ethylene and jasmonic acid promote leaf senescence in tomatoes.

Key Message

Our findings not only confirmed that *SlERF.F5* can regulate tomato leaf senescence, but also provided new insights into the mechanism of how ethylene and jasmonic acid promote tomato leaf senescence.

1. Introduction

Leaf senescence is a necessary process in the growth and development of plants. It's an active and controllable process. During the senescence process, plant leaf cells have undergone tremendous changes in the structure, metabolism, and gene expression in a programmed manner (Lim, Kim and Nam 2007, Zhao et al. 2018). Decomposition of the chloroplast is one of the earliest and most noticeable changes in cell structure. In terms of metabolism, the main differences are the loss of photosynthesis and the hydrolysis of macromolecular substances, such as proteins and nucleic acids (Woo et al. 2013, Lim and Nam 2005). These hydrolyzed molecules are transported to the developing fruits and seeds, which are very important for plant survival and sustainability in annual plants (Woo et al. 2013, Bresson et al. 2018).

Leaf senescence is the last step of plant leaf growth and development, and it is affected by growth, hormones, and external environment, such as age, darkness, drought, and pathogen attacks (Lim et al. 2007). Among the many factors that affect leaf senescence, plant hormones are essential. According to reports, leaf senescence is affected by the main plant hormones. Among them, ethylene, methyl jasmonate (MeJA), abscisic acid (ABA), salicylic acid (SA), and brassinosteroids can promote

senescence. However, cytokinins (CKs), gibberellin acid (GA), and auxin can inhibit senescence (Gan and Amasino 1997, Jibran, Hunter and Dijkwel 2013). But so far, the potential regulatory mechanism of leaf senescence and the role of hormones have not been fully elucidated.

As we all know, ethylene is the most common and very crucial plant hormone. It participates in many growth and development processes, including cell elongation, seed germination, flowering, fruit maturation, organ senescence, and response to stress (Johnson and Ecker 1998). It has a positive regulatory effect on fruit ripening and organ senescence (Abeles et al. 1988). However, the cause of leaf senescence does not necessarily require the presence of ethylene. Ethylene can only regulate the time of senescence, and treating young leaves with ethylene will not cause senescence (Jing et al. 2002, Jing et al. 2005, Podzimska-Sroka et al. 2015). According to reports, many transcription factors related to ethylene play an essential role in plant senescence. For example, in *Arabidopsis* dark-induced leaf senescence experiments, *NO* can regulate *EIN2* (a transcription factor for ethylene signaling) to promote senescence (ETHYLENE INSENSITIVE 2) (Niu and Guo 2012). *EIN3*, a key transcription factor for ethylene signaling, is constitutively overexpressed or temporarily activated to accelerate leaf senescence symptoms (Li et al. 2013). Besides, jasmonic acid (JA) also plays a central role in the senescence of plant leaves induced by darkness. It can influence the expression of various genes to promote senescence (He et al. 2002, Jung et al. 2007). For example, during JA-induced leaf senescence, the level of WRKY57 protein is reduced, and the *wrky57* mutant produces a typical leaf senescence phenotype in Arabidopsis, WRKY57 can interact with JAZ4/8 (Jiang et al. 2014). In JA-induced leaf senescence, *Dof2.1* acts as an enhancer. It mainly enhances leaf senescence by promoting *SlMYC2* (the helix-loop-helix transcription factor) expression (Zhuo, Sakuraba and Yanagisawa 2020). Also, *SlMYC2* regulating the root growth and the defense of pathogen infections regulated by JA, and it plays a positive regulatory role in JA-induced leaf senescence (Song et al. 2017). Moreover, *SlMYC2* is activated by jasmonic acid and can inhibit the activity of *EIN3* (Zhang et al. 2014). In the research related to ethylene and jasmonic acid, a group of JAZ proteins (JAZ1, JAZ3, and JAZ9) can directly bind to *EIN3* and *EIL1* involved in ethylene signaling (Zhu et al. 2011). According to reports, ethylene and jasmonic acid have been found to coordinate (cooperatively or antagonize) plant growth and development and respond to stress (Li and Guo 2007). However, the molecular mechanism of their coordination has been relatively little studied.

Senescence is caused by a complex network of gene regulation (Nam 1997). Many studies have shown that through differential expression methods, through the identification and characterization of senescence-related mutants and hundreds of senescence-related genes (SAGs), there have been many advances in the understanding of leaf senescence at the molecular level (Buchanan-Wollaston et al. 2003, Li et al. 2012). With the in-depth study of leaf senescence, more and more genes of the transcription factor family have been confirmed to be related to senescence. These transcription factor families mainly include WRKY, NAC, MADS, MYB, bZIP and bHLH (Li et al. 2013). Studies have shown that more than 200 transcription factor families are involved in the regulation of leaf senescence, which indicates that leaf senescence is controlled by a complex transcriptional regulatory network (Liu et al. 2011, Li et al. 2012, Li et al. 2013). For instance, darkness promotes the transcription of *AtWRKY22*, which is involved senescence regulation (Zhou, Jiang and Yu 2011). In promoting leaf senescence,

ANAC046 regulates Chl catabolism genes and senescence-related genes in *Arabidopsis* (Oda-Yamamizo et al. 2016).

The AP2/ERF (APETALA2/Ethylene Response Factor) family is one of the largest plant transcription factors, with approximately 140–280 members in various plants. The functions of many genes in this family have been thoroughly studied. It mostly related to planting growth and development, biological, and abiotic stresses, and fruit maturation, mainly by controlling the response to various plant hormones (ethylene, abscisic acid, and jasmonic acid). They can represent the diversity and specificity of ethylene reactions (Li et al. 2018, Liu et al. 2016, Nakano et al. 2006). The ethylene response factor (ERF) family belongs to the AP2/ERF superfamily, characterized by a highly conserved AP2 DNA binding domain consisting of 60–70 amino acid residues (Ohmetakagi and Shinshi 1995, Sakuma et al. 2002). The ERF domain comprises three β-sheets and an α-helix, where β-sheets can recognize various cis-acting elements. The α-helix may be involved in the interaction with other transcription factors or DNA. Based on the differences in the amino acid sequence of the ERF domain, ERF proteins are divided into four categories (Tournier et al. 2003). Among them, the class II ethylene-responsive transcription factor (ERF) contains a conserved repressor domain L/FDLNL/F(x)P at the C-terminus, which is called the ERF-related amphiphilic repressor (EAR) motif or CMVIII-1 motif (Nakano et al. 2006). The unique feature of this family is the ability to recognize GCC-box (AGCCGCC) (Ohmetakagi and Shinshi 1990). GCC-Box is the best target for ERFs class proteins, and class II putative repressor ERFs are more flexible than other proteins (Tournier et al. 2003). ERF generally acts as a transcriptional activator, but ERF containing EAR acts as a GCC-mediated transcriptional repressor (Ohta et al. 2001). ERF transcription factors can interact with genes in other hormone signaling pathways. Research proves that ethylene and MeJA can activate the expression of *AtERF1* separately or cooperatively, indicating that *AtERF1* plays an essential role in the signal transduction pathway of ethylene and jasmonic acid (Lorenzo et al. 2003).

In recent years, studies have shown that AP2/ERF family genes are also related to senescence, *SlERF36* overexpression can promote flowering and senescence (Upadhyay et al. 2013). Although AP2/ERF family genes are related to senescence, its research is still sparse and not deep enough. Here, a gene from the AP2 family, *SlERF.F5*, was isolated from tomato (Solanum lycopersicum, Mill. cv. Ailsa Craig, AC^{++}). In this article, a study of *SlERF.F5* gene silencing was conducted to clarify the function of *SlERF.F5* in tomatoes. Studies have shown that under normal and dark and hormone-treated conditions, the *SlERF.F5*-RNAi lines showed accelerated leaf senescence. The morphological, biochemical, and molecular comparisons of WT and *SlERF.F5*-RNAi lines further confirmed that silencing *SlERF.F5* can promote senescence. The yeast two-hybrid experiment shows that SlERF.F5 can interact with SlMYC2, downstream of the JA receptor, *SlMYC2* positively regulates leaf senescence induced by JA, thereby promoting senescence. In short, *SlERF.F5* may promote senescence under the action of ethylene and MeJA, which provides new ideas for studying the role of ethylene and MeJA in leaf senescence.

2. Materials And Methods

2.1 Plant materials and growth conditions

The wild-type (WT) tomato (Solanum lycopersicon Mill. cv. Ailsa Craig, AC^{++}) and *SlERF.F5*-RNAi, *SlERF.F5*-overexpressing transgenic lines were used in this study. Tomato growth conditions: 16-h day (28°C) /8-h night (18°C) cycle, greenhouse. To detect the response of *SlERF.F5* to plant hormones, 35-day-old WT tomato seedlings were treated with 50 μM abscisic acid (ABA), 50 μM indole 3-acetic acid (IAA), 50 μM Jasmonic acid (MeJA), 100 μM gibberellin (GA3), 50 μM 1-aminocyclopropane-1-carboxylate (ACC) and distilled water, respectively. Three treatments were performed for each hormone, with three biological replicates. After 0, 1, 2, 4, 8, 12, and 24 hours of treatment, the third leaf was collected from the top of the wild tomato seedlings. To examine the specific expression of *SlERF.F5* in tomato, various tissue samples of WT tomato were collected. These tissues include: roots (RT), stems (ST), young leaves (YL), mature leaves (ML), senescent leaves (SL), flowers (FL), immature green (IMG), mature green (MG), break (B), 4 days after break (B + 4), and 7 days after break (B + 7). All these samples were immediately wrapped in foil, frozen with liquid nitrogen and stored in a -80°C refrigerator.

2.2 Construction of *SlERF.F5*-RNAi and *SlERF.F5*-overexpression vectors and plant transformation

To obtain the *SlERF.F5*-RNAi transgenic lines, the 485 bp fragment of *SlERF.F5* was amplified with the primers SlERF.F5-RNAi-F1/R1 (Supplementary Table S1), and ligated into the pBIN19 vector to form the *SlERF.F5*-RNAi vector, which can produce hairpin SlERF.F5-specific DNA fragments. The vector construction process was performed according to our previous report (Zhou et al. 2019). To construct the *SlERF.F5*-overexpressing vector, used primers SlERF.F5-full-F2/R2 (Supplementary Table S1) to amplify the full-length *SlERF.F5* cDNA. The amplified products were digested with XbaI/SacI and linked to the plant binary vector pBI121 under the control of the CaMV 35S promoter at XbaI/SacI restriction sites. The constructed vector was transformed into tomato cv. Ailsa Craig by *Agrobacterium tumefaciens* LBA4404 strain through the freeze-thaw method. Finally, transgenic lines were selected by kanamycin (50 mg/L), and confirmed by PCR using NPTII-F/R primers (Supplementary Table S1).

2.3 Total RNA extraction and Quantitative reverse-transcription-PCR analysis

Total RNA was extracted from stored samples using Trizol reagent (Invitrogen, Shanghai, China). RNA extraction method based on our previous research (Xie et al. 2014).

Quantitative reverse-transcription-PCR (qRT-PCR) was performed by using a CFX96™ RealTime System (Bio-Rad, USA). PCR reaction system: 5 μL enzyme solution (2 × GoTaq®qRT-PCR Master Mix, Promega), 3.5 μL distilled water, 0.5 μL primer pair (10 mM), and 1 μL cDNA. PCR reaction program: 95°C for 3 minutes, then at 95°C for 15 seconds, and Tm (the most suitable temperature) for 45 seconds for 40 cycles. *SlCAC* with relatively stable expression was selected as the internal reference (Nicot et al. 2005), and the expression level of the gene was analyzed using the $2^{-\Delta\Delta CT}$ method. All samples were repeated three times. The primers used in this experiment were listed in Supplementary Table S1.

2.4 Determination of leaf senescence induced by age, darkness, ethylene, and MeJA

For age-dependent leaf senescence, 10-week-age tomato leaves of WT and *SlERF.F5*-RNAi lines were sampled, and their chlorophyll contents were measured, respectively. For dark-induced senescence experiments, we selected 10-week-age plants, and detached mature leaves of the same location from WT and *SlERF.F5*-RNAi lines, and place them on the filter paper containing 3 mL of distilled water at the bottom of a 150 mm Petri dishes. Place it in a dark environment and keep it at 22°C. Take samples at 0 d, 5 d, and 7 d, respectively.

In the experiment of hormone and darkness-induced leaf senescence, mature leaves of WT and RNAi tomato seedlings for 10-week-age were collected and placed on a filter paper in a Petri dish with a diameter of 150 mm. Then 3 mL of distilled water, 100 μM ACC, 50 μM MeJA, or 10 μM $AgNO_3$ were added into the dish, respectively, covered, and placed in a dark environment at 22°C.

2.5 Measurement of total chlorophyll and carotenoids content

To detect the age, darkness, and hormone treatment of the leaf senescence of *SlERF.F5*-RNAi and WT lines, the contents of chlorophyll and carotenoid were detected. Weigh the fresh leaves, grind them thoroughly with liquid nitrogen, and extract with 80 % acetone. The specific experimental process and calculation method are described by Wellburn et al. (Wellburn 1994). Each experiment was repeated three times in biology, and each determination technique was repeated three times.

2.6 Measuring malondialdehyde (MDA) and electrolytic leakage

To detect the malondialdehyde (MDA) content, the fresh leaves were thoroughly ground with liquid nitrogen, 0.2 g was weighed into a centrifuge tube, and 4 mL of 10 % trichloroacetic acid (TCA) was quickly added, mixed, and centrifuged at 15,000 g for 5 minutes. Pipet 1 mL of the supernatant into a new centrifuge tube, and then add 4 mL of 10 % trichloroacetic acid solution containing 0.5 % thiobarbituric acid (TBA). After mixing, incubate at 95°C for half an hour, then place it in an ice-water mixture to stop the reaction. After 10,000 rpm, 10 min, and measure the absorbance of the supernatant at 532 nm and 600 nm. Repeat three times for each sample. This method is described by Sanjaya et al. (Sanjaya et al. 2008) and Zhang et al. (Zhang et al. 2009).

For the detection of electrical conductivity, take tomato leaves of comparable size (try to ensure the integrity of the leaves, with few stems and nodules), rinse with tap water and rinse with distilled water three times, absorb the surface moisture with filter paper, and take a punching method, avoiding the main vein, each 20 round leaves were placed in a graduated test tube of 50 mL, added with 20 mL of distilled water, and soaked at 28°C for 12 h. Measure the conductivity of the leaching solution (R1) with a conductivity meter, then place the test tube in boiling water for 30 minutes, and cool to 28°C, shake it up,

and measure the conductivity of the leaching solution again (R2), relative electrical conductivity = R 1/R 2*100 %.

2.7 Superoxide dismutase (SOD)

For the determination of superoxide dismutase (SOD) activity, the WST method was used, and the operation steps refer to the WST method kit instructions.

2.8 Yeast two-hybrid

The open reading frame of *SlERF.F5* was amplified by PCR using primers SlERF.F5-F and SlERF.F5-R (Table 1S). The PCR product was digested with *Sma*I and *Bam*HI, and cloned into the pGBKT7 bait vector to obtain the vector SlERF.F5-pGBKT7. At the same time, using the primer pairs SlMYC2-F and SlMYC2-R, the open reading frames of *SlMYC2* were amplified by PCR (Table 1S), and digested with *Sma*I and *Bam*HI, cloned into the pGADT7 vector to obtain the vectors SlMYC2-pGADT7. Then the constructed vectors were transferred into Y2Hgold, respectively. Yeast two-hybrid with bait was plated on SD medium lacking Trp, Leu, and SD medium lacking Trp, His, Ade, Leu to test the self-activation of SlERF.F5-pGBKT7, SlMYC2-pGADT7. SlERF.F5-pGBKT7 and SlMYC2-pGADT7 were co-transformed into Y2Hgold. Plated it on SD medium lacking Trp, Leu, and culture it upside down for three days. Pick single colonies on SD medium lacking Trp, His, Ade, Leu, and culture it upside down for 1–2 days, and use X-a-Gal (QDO/X) to judge whether SlERF.F5 can interact with SlMYC2.

2.9 Statistical analysis

Data were presented as mean ± standard deviation. A significant difference between transgenic lines and WT were analyzed using the Student's t-test (*, $P < 0.05$).

3. Results

3.1 Sequence and phylogenetic tree analyses of SlERF.F5

Based on the sequence in the Tomato Genome Database (https://solgenomics.net, accession No. Solyc10g009110), *SlERF.F5* contains 1466 base pairs (bp) encoding a putative protein of 222 amino acids. *SlERF.F5* is named by Liu (Liu et al. 2016). Multi-sequence alignment of proteins based on DNAMAN. This protein contains a typical AP2-domain. AP2 domains are typically characterized by three β-sheets and one α-helix (Fig. 1a). Based on previous studies, *SlERF.F5* belongs to the class II putative repressor ERFs.

The phylogenic tree was calculated by MEGA (Molecular Evolutionary Genetics Analysis) version 5.0. Phylogenetic analysis based on selected full-length protein sequences of ERF showed that SlERF.F5 is most related to NtEREBP5, followed by AtERF4, AtERF3, SodERF3 (Fig. 1b). Currently, there is no research on the *NtEREBP5* gene. Regarding *AtERF4*, it acts as a class II repressor and can be induced by ethylene, jasmonic acid, and abscisic acid (Yang et al. 2005). Besides, in the case of iron deficiency, AtERF4 can regulate the expression of these two genes by combining the promoters of *AtCLH1* and *AtITR1*, thereby

playing a negative regulatory role in the stress process (Liu et al. 2017a). SodERF3 belongs to class II putative repressor ERF and can be bind to GCC-box. Overexpression of *SodERF3* improves tolerance to drought and salt in tobacco (Trujillo et al. 2008). In the previous research of *SlERF.F5*, it is mainly related to stress, but its role in tomato growth and development has not been reported.

3.2 Expression pattern analysis of *SlERF.F5*

To clarify the role of *SlERF.F5* in tomato growth and development, the accumulation of its transcripts in various tissues is quantified by qRT-PCR. Figure 2a shows that the expression level of *SlERF.F5* in leaves, MG, and B + 4 fruit is relatively high, while it is low in other tissues. The specific expression of *SlERF.F5* suggests that it may play a role in leaves and fruits. We sought to study further the role of *SlERF.F5* in leaf growth and development. We examined young tomato leaves (Y), mature leaves (M), early senescent leaves (leaf yellowing area > 25%, EL) and late senescent leaves (leaf yellowing area > 50%, LS) (Fig. 2b). The mRNA level detected by qRT-PCR confirmed that the transcription level of *SlERF.F5* gradually decreases during leaf development and senescence (Fig. 2c). We also test two photosynthesis genes *Cab7* (chlorophyll/binding protein 1), *RBCS* (ribose bisphosphate carboxylase small chain), and *RAV1* (related to ABI3/VP1), and their expression levels gradually decreased as the leave senescence (Fig. 2d-f). As a positive control for senescence, *SlSAG12* is a widely used molecular marker for leaf senescence, expressed explicitly in senescent leaves (Fig. 1g). Here, only to show the relative expression of genes, so the expression level of WT is set to one in the qRT-PCR results. The expression level of *SlERF.F5* gradually decreases with leaf senescence, which indicates that silencing the expression level of *SlERF.F5* might affect leaf senescence.

Given that *SlERF.F5* is a downstream receptor component of the ethylene signal transduction pathway. It is closely related to ethylene, to further confirm whether *SlERF.F5* is affected by other hormones, we used qRT-PCR to examine the expression patterns of *SlERF.F5* under different hormone treatments. Figure 2h shows that the accumulation of *SlERF.F5* transcripts increases rapidly to the maximum after one hour of hormone treatment (such as IAA, ABA, MeJA, GA3, and ACC). With the increase of treatment time, the expression level of *SlERF.F5* gradually decreases, indicating that hormone treatment may affect the expression of *SlERF.F5* in a short time.

3.3 Silencing of *SlERF.F5* accelerates the senescence of tomato leaves

To further clarify the effect of *SlERF.F5* on tomato growth and development and the role of other phytohormones, we constructed the *SlERF.F5*-RNAi vector and obtained the transgenic lines through the *Agrobacterium*-mediated genetic transformation method. Examination of its silencing efficiency (Fig. 3a) shows that compared with the WT, the expression of *SlERF.F5* in the leaves of the five transgenic lines was significantly reduced by 94–98%. The best silencing efficiency lines are 5, 13, and 15. Because the 5 and 15 lines harvested few seeds, they could not support the next research, so we chose the 10, 13, and 16 lines, called RNAi10, RNAi13, and RNAi16, and conduct further research. At the age of 6 weeks of tomato seedlings, the silent lines show premature senescence (Fig. 3b), and Fig. 3c shows the leaves of

the same part of the WT and *SlERF.F5*-RNAi lines. The leaves of RNAi lines appear yellow, while the leaves of WT are still greener. To see the color and shape of each leaf more clearly, the leaf is split and taken a photo (Fig. 3d). Because of the senescent phenotype of the leaves, we test their chlorophyll content. The 6-week-old tomato plants were labeled as the first leaf, the second leaf, and so on, until the sixth leaf. Detect the chlorophyll content of each leaf. From Fig. 3e, we can find that the chlorophyll content of WT leaves is slightly higher than that of RNAi leaves at different leaf ages. Further statistics on the senescence time of tomato leaves reveal that the senescence time of RNAi lines is earlier than that of WT (Fig. 3f). Compare with the wild type, the leaf senescence time of RNAi lines is advanced by about one week. These data demonstrated that silencing of *SlERF.F5* could promote the senescence of tomato leaves.

3.4 Silencing of *SlERF.F5* promotes dark-induced leaf senescence

Dark-induced leaf senescence is a common way to study senescence (Li et al. 2013). To further study the role of *SlERF.F5*-RNAi in leaf senescence, we take mature leaves for in vitro experiments. As shown in Fig. 4a, the edge of leaves of the *SlERF.F5*-RNAi lines start to become yellow when treating for five days in the dark, and at seven days, some leaves turned almost completely yellow, and the edges of the remaining leaves had turned yellow, whereas, the leaves of the WT are still green after seven days treatment. To further confirm the phenotype of senescence, we measure some physiological indicators related to leaf senescence. First, detecting the chlorophyll content, we find that at day 0, the chlorophyll content of the RNAi lines is higher than that of the WT. This may be because the leaves of the RNAi lines are more mature, and the nutritional status was better than that of the WT. However, with the dark treatment time increase, the chlorophyll content of *SlERF.F5*-RNAi lines leaves gradually decrease. It is significantly lower than that of WT at 7 d, while the chlorophyll content of WT leaves only decreased slightly during the treatment (Fig. 4b). MDA is an important indicator of membrane damage (Sanjaya et al. 2008). Compared with WT, the MDA content in the leaves of the *SlERF.F5*-RNAi lines is significantly higher. When treating in the dark for 5d to 7d, the rate of increase of MDA content in the leaves of RNAi lines is slightly higher than that of WT (Fig. 4c). Further, the electrical conductivity is also an indicator of the membrane damage of the blade. At 0 d, the electrical conductivity of the WT blade is slightly higher than that of the *SlERF.F5*-RNAi blade. This may be due to the damage received by sampling, but at 7 d, the electrical conductivity of RNAi lines leaves is higher than that of WT leaves (Fig. 4d). Besides, the activity of superoxide dismutase (SOD) is also an indicator of the degree of damage to the cell membrane. At 0 d, the SOD activity of the RNAi lines' leaves is lower than that of WT (Fig. 4e), which may be caused by damage to the leaves. however, Fig. 4e shows that after five days, compared with WT, the SOD activity of *SlERF.F5*-RNAi leaves is significantly lower. These results indicate that silencing of *SlERF.F5* gene leads to premature leaf senescence under dark conditions.

3.5 Silencing of *SlERF.F5* affects the expression of chlorophyll, ethylene, and jasmonic acid related genes

To reveal the possible molecular mechanism in WT and *SlERF.F5*-RNAi dark-treated senescent leaves for five days, qRT-PCR is used to detect some genes related to chlorophyll. The transcription levels of chlorophyll genes, including magnesium chelatase H subunit (*CHLH*), Mg protoporphyrin IX methyltransferase (*CHLM*), protochlorophyllide reductase (*POR*), and chlorophyllide an oxygenase (*CAO1*), pheophytin pheophorbide hydrolase (*PPH*), STAY-GREEN Protein (*SGR1*), *AUREA* and the genomes uncoupled 4 (*GUN4*), are significantly down-regulated in *SlERF.F5*-RNAi transgenic lines (Fig. 5a-h). Among the genes, *SGR1* can induce chlorophyll degradation, which is down-regulated in RNAi lines. However, the chlorophyll content of the leaves of RNAi lines is lower than that of WT, indicating that the decline of chlorophyll content is regulated by a network of many genes. The down-regulation of a single *SGR1* gene does not affect the change of chlorophyll content. Besides, the expression level of ribulose bisphosphate carboxylase small chain (*RBCS*) and light-harvesting protein complex 1 (*LHCA1*) is also down-regulated in the *SlERF.F5*-RNAi lines (Fig. 5i, j). A molecular marker widely used for leaf senescence, *SlSAG12*, can be used as a positive control. Figure 5k shows that *SlSAG12* is significantly upregulated in the *SlERF.F5*-RNAi lines compared to WT. According to these results, we speculate that silencing of *SlERF.F5* gene affects the expression of some genes in the pathway of chlorophyll, thereby reducing the chlorophyll content, which is one of the reasons for leaf senescence.

To investigate the ethylene's role in leaf senescence of the silenced *SlERF.F5* lines, some of the ethylene signal synthesis pathway genes are examined. Expression of 1-aminocyclopropane-1-carboxylate oxidase 1 (*ACO1*), 1-aminocyclopropane-1-carboxylate synthase 2 (*ACS2*), 1-aminocyclopropane-1-carboxylate synthase 4 (*ACS4*) are up-regulated in the *SlERF.F5*-RNAi lines compared to WT (Fig. 6a-c), indicating that silencing of *SlERF.F5* may increase the ethylene content, thereby promoting leaf senescence. Since MeJA plays a positive regulatory role in leaf senescence, and recent years, studies have pointed out that SlMYC2 can regulate plant growth and development through physical interaction with EIN3. Thus, the expression levels of MeJA signaling pathway transcription factors are detected. JASMONATE ZIM-domain (JAZ) genes (in. *JAZ1*, *JAZ2*, *JAZ4*, *JAZ7*, *JAZ11*) are up-regulated in the *SlERF.F5*-RNAi lines (Fig. 6d-h). Besides, the expression of *SlMYC2*, a transcription factor downstream of the JA receptor, in the leaves of RNAi lines is higher than that of WT (Fig. 6i). *COI1* is an essential regulator of JA-induced leaf senescence. Figure 6g shows that its expression in the leaves of *SlERF.F5*-RNAi lines is also higher than that of WT. The above experimental results indicated that suppression of *SlERF.F5* gene could increase the gene expression of ethylene biosynthesis, jasmonic acid signal transduction, and receptor downstream transcription factors, which may increase the content of ethylene and jasmonic acid and cause leaf senescence.

3.6 Ethylene and jasmonic acid accelerate the senescence of *SlERF.F5*-RNAi leaves in dark conditions

To further clarify the role of ethylene and jasmonic acid in promoting leaf senescence, we did a triple reaction induced by ACC and a sensitivity test of MeJA. 0 μM, 5 μM, 10 μM ACC (1-aminocyclopropane-1-carboxylic acid) is used to treat the germinated seeds of WT and *SlERF.F5*-RNAi lines. After five days of

cultivation in the dark, *SlERF.F5*-RNAi lines show a slightly lower length of hypocotyls and root, the lighter weight of seedling than the WT (Supplementary Fig. S1a-d), suggesting that silenced-*SlERF.F5* seedling is more sensitive to ACC. Besides, we have noticed that at 0 μM, compared with WT, the seedling root length of the RNAi lines is shorter, and the seedling weight is heavier, indicating that under normal circumstances, the seedling growth of the RNAi lines is better than that of WT. To further confirm this phenotype, the expression levels of *ACO1* and *ACS2* in seedling hypocotyls are analyzed by qRT-PCR, and results show that after treatment with ACC (10 μM), the transcripts of the two genes in silenced-*SlERF.F5* seedlings are higher than those in WT (Supplementary Fig. S1e,f). In the MeJA sensitivity experiment, After seven days of culture under normal conditions, the length of the hypocotyl, root, and seedling weight of the RNAi lines are lower than those of the WT (Supplementary Fig. S2a-d). These results indicate that silenced *SlERF.F5* lines are more sensitive to MeJA.

To verify the role of ethylene and jasmonic acid in leaf senescence, we use detached leaves to carry out hormone-induced senescence experiments. We added hormone treatment (ACC and MeJA) in the dark-induced experiment. AgNO3 is an inhibitor of ethylene action in plants. The purpose of treatment with AgNO3 and MeJA is to test the effect of jasmonic acid on leaf senescence in the absence of ethylene. The results show that after seven days of hormone treatment in dark conditions, the leaves of the *SlERF.F5*-RNAi lines are yellower than WT (Fig. 7a). Compared with WT, the chlorophyll content of *SlERF.F5*-RNAi is significantly lower (Fig. 7b), and the MDA content is higher (Fig. 7c). The results demonstrate that both ethylene and jasmonic acid treatments can promote leaf senescence of *SlERF.F5*-RNAi lines under dark conditions. Also, jasmonic acid alone can also promote leaf senescence of *SlERF.F5*-RNAi lines, indicating that ethylene and jasmonic acid synergistically promote leaf senescence.

3.7 Overexpression of *SlERF.F5* may delay dark-induced leaf senescence

To further verify the function of *SlERF.F5* on the leave senescence, we also constructed a *SlERF.F5* overexpression vector, transformed it into tomato cotyledon, and obtained transgenic lines overexpressing *SlERF.F5*. qRT-PCR is used to detect the expression level of *SlERF.F5*. As shown in Fig. 8a, we selected lines 1, 5, and 6 (OE1,OE5, and OE6) with higher expression efficiency for the next experiment. Similarly, a dark-induced leaf senescence experiment is conducted. The leaves of 10-week-old seedlings of WT, *SlERF.F5*-RNAi, and *SlERF.F5*-OE lines are harvest, respectively, and the dark-induced senescence experiment is carried out in the same way as above. After five days, the leaves of *SlERF.F5*-RNAi plants began to turn yellow, while no noticeable color change occurred in the leaves of WT and *SlERF.F5*-OE lines (Fig. 8b). Compared with WT, the chlorophyll and carotenoid content of *SlERF.F5*-OE lines are slightly higher (Fig. 8c and 8d). The chlorophyll content of RNAi and overexpressing lines and WT for five days after dark treatment are sorted together for comparison (Fig. 8e). The results showed that the chlorophyll content of the leaves of the *SlERF.F5*-OE lines is slightly higher than that of the WT after dark treatment for five days, but at five days the greenness of WT leaves remained more than that of *SlERF.F5*-RNAi lines, and the total chlorophyll content is higher. Also, after five days the SOD activity of RNAi leaves is lower

than that of WT, whereas the *SlERF.F5*-OE lines leaves show higher SOD activity than that of WT (Fig. 8f). The above results suggest that suppression of *SlERF.F5* can promote leaf senescence, and overexpression of *SlERF.F5* might inhibit leaf senescence.

3.8 SlERF.F5 interacts with SlMYC2

Based on the above research results, we have obtained that *SlERF.F5*-RNAi lines displayed promoted leaf senescence under normal growth conditions and under the treatment of dark, ethylene, and jasmonic acid. Given that *SlMYC2* is a vital transcription factor downstream of the jasmonic acid receptor and it has a direct relationship with aging, its interaction with *SlERF.F5* is the first choice for studying the regulation of aging by ethylene and jasmonic acid. Therefore, we selected SlMYC2 protein to perform yeast two-hybrid experiments. Results showed that the yeast cells co-expressing SlERF.F5-BD and SlMYC2-AD could grow on the quadruple dropout medium (SD/-Leu-Trp-His-Ade), the same as the yeast cells carrying pGADT7-T and pGBKT7-53 (Positive control), indicating that there is a physical interaction between SlERF.F5 and SlMYC2 (Fig. 9). The results show that SlERF.F5 can interact with SlMYC2 and play an essential role in leaf senescence induced by ethylene and jasmonic acid.

Discussion

Discussion

SlERF.F5 belongs to the AP2/ERF family, studies of this family gene major in plant growth, biological and abiotic stress, and fruit ripening. In Arabidopsis, *AtERF4* and *AtERF8* belong to class II ERF. *LeERF3b* accumulates before the fruit matures and then declines sharply, and drought and low temperature can significantly induce the expression of *LeERF3b* (Chen, Hu and Grierson 2008). Overexpression of *SlERF.B3-SRDX* leads to a significant delay in fruit ripening time, increased fruit softening, and reduced pigment accumulation (Liu et al. 2014). Overexpression of *SlERF5*, as a class III ERFs protein, can increase tolerance to drought and salt (Pan et al. 2012). In Arabidopsis thaliana, ethylene response factor 53 (*AtERF53*) affects the expression of drought-responsive genes by combining GCC-box and, or dehydration response elements in the downstream gene promoter, and *AtERF53* plays a negative regulatory role in drought stress by affecting the transcriptional activity of *RGLG2* (Cheng et al. 2012). Under iron deficiency conditions, *AtERF72* can affect the expression of chlorophyll-degrading genes pheophytin an oxidase (*PAO*) and chlorophyllase (*CLH1*), and *ERF72* can directly bind to the promoter regions of *IRT1*, *HA2*, and *CLH1*, thereby regulating the plant's lack response to iron stress (Liu et al. 2017b). *TERF2/ LeERF2* plays a positive regulatory role in ethylene biosynthesis, and it can enhance the freezing resistance of plants (Zhang and Huang 2010). *JERF3* can activate the expression of *DRE* and *CE1* genes containing GCC-box, thereby reducing the accumulation of ROS and enhancing the adaptability of tobacco to drought, low temperature, and high salt (Wu et al. 2008). According to the function of family genes and previous studies, *SlERF.F5* may also affect plant growth, stress, and fruit ripening.

In this study, along with the growth and development of leaves, the expression level of *SlERF.F5* gene gradually decreases (Fig. 2c). The expression pattern of *SlERF.F5* gene was similar to *Cab7*, *RBCS*, *RAV1*, but it was opposite to the expression pattern of *SlSAG12*. Whether it was age-dependent senescence (Fig. 3b), darkness-induced senescence (Fig. 4a), or hormone-induced senescence (Fig. 7a), the leaves of the *SlERF.F5*-RNAi lines showed earlier senescence than WT. These experimental results indicated that *SlERF.F5* plays a negative role in leaf senescence. Besides, we also created the *SlERF.F5*-OE tomato lines. In the dark-induced senescence experiment, compared with WT plants, the chlorophyll content, SOD activity, and carotenoid content of the *SlERF.F5*-overexpression lines were slightly higher, and no apparent yellowing phenomenon was observed (Fig. 8b-f). This indicated that overexpression of *SlERF.F5* would not promote senescence. On the contrary, senescence may be delayed. Chlorophyll content reflects the senescence of the leaves. Similarly, compared with WT plants, we found that the chlorophyll content of *SlERF.F5*-RNAi lines at 5 d and 7 d were significantly lower (Fig. 4b). Next, we measured the expression levels of some genes for chlorophyll (*CHLH*, *CHLM*, *POR*, *CAO1*, *GUN4*, *PPH*, *SGR1*, *AUREA*), and light-responsive gene (*RBCS*, *LHCA1*). In researches related to chlorophyll biosynthesis and degradation genes, compared with WT plants, *PPH*, *PAO*, *RCCR*, *SGR1* in the *SlOFP20*-OE line was significantly increased. Overexpression of *SlOFP20* can regulate chlorophyll accumulation and leaf senescence (Zhou et al. 2019). The expression levels of *RBCS1A* and *CAB1* were examined to investigate the relationship between *EIN3* and leaf senescence (Li et al. 2013). During leaf senescence, the expression of *SlNAP2* increased. *SlNAP2* can activate the expression of *SlSGR1* and *SlPAO* to regulate senescence (Ma et al. 2018). According to previous research, the expression changes of chlorophyll synthesis and metabolism genes were mostly related to leaf senescence. In this article, these genes were down-regulated in the senescent leaves of the *SlERF.F5*-RNAi lines, which clarified the mechanism of leaf senescence from a physiological and molecular level.

Among the main hormones that affect leaf senescence, ethylene, as promoters of leaf senescence, play a signicant role in age and darkness-induced senescence. During darkness-induced leaf senescence, ethylene-insensitive mutants (*ein2*/*ore3*) act as senescence promoting factors through transcriptional regulation of stress-related responses (Kim et al. 2018). In the *ein3 eil1* double mutant, ethylene inhibits the expression of *NYE1*, *NYC1*, and *PAO* containing GCC-box, and EMSA results indicate that *EIN3* can directly bind *NYE1*, *NYC1*, and *PAO* promoters and play central a role in ethylene-mediated leaf senescence (Qiu et al. 2015). Therefore, we supposed that ethylene might be responsible for the leaf senescence of *SlERF.F5*-RNAi lines. In this study, the expression levels of *ACO1*, *ACS2*, *ACS4*, the ethylene biosynthetic pathway genes in the *SlERF.F5*-RNAi lines were significantly increased compared with WT, suggesting that suppression of *SlERF.F5* might induce the biosynthesis of ethylene, thereby promoting senescence.

Jasmonic acid is also a promoting factor of leaf senescence, and changes in the expression of jasmonic acid-related genes are critically related to leaf senescence. In research on leaf senescence, the expression of *JAZ7* was up-regulated in darkness-induced senescence. The *jaz7* mutant showed a large area of yellowing of the leaves. In addition, the double mutants of *jaz7 SlMYC2* and *jaz7 coi1* showed delayed leaf senescence. In conclusion, JAZ7 protein as a regulator of dark-induced leaf senescence (Yu et al.

2016). JAZ4 and JAZ8 can physically interact with WRKY57 and play a negative regulatory role in MeJA-induced leaf senescence (Jiang et al. 2014). In MeJA-induced senescence experiments, *MYC5* overexpressing transgenic plants showed early leaf senescence phenotypes, including reduced chlorophyll content. It enhanced JA-regulated senescence-related gene expression (*SAG13*, *SEN4*, *SAG113*, and *SAG29*) and photosynthesis genes (*RBCS* and *CAB1*) (Song et al. 2017). In the mechanism of MeJA-induced leaf senescence, COI1-dependent JA inhibition was considered to be very important (Shan et al. 2011). The experimental results showed that the expression levels of *JAZ1*, *JAZ2*, *JAZ4*, *JAZ7*, *JAZ11* in the jasmonic acid signaling pathway in the *SlERF.F5*-RNAi lines were significantly increased compared with WT (Fig. 6). Besides, the expression of downstream transcription factors of JA receptor (*SlMYC2*) and *COI1* gene was also increased compared with WT (Fig. 6). According to previous reports, increased expression of these genes could promote leaf senescence, may also improve the jasmonic acid content, which may also be one of the reasons for the early senescence of the leaves of the *SlERF.F5*-RNAi lines.

In the hormone-induced senescence experiment, exogenous addition of ACC and MeJA can induce senescence of *SlERF.F5*-RNAi leaves. Moreover, the exogenous addition of $AgNO_3$ (an ethylene inhibitor) and MeJA can also induce senescence of *SlERF.F5*-RNAi leaves. This result indicated that MeJA could also induce senescence in the absence of ethylene (Fig. 7a-c). According to the above results, we can speculate that ethylene and jasmonic acid play a synergistic role in the process of leaf senescence. In the hormone sensitivity experiment, the tomato seedlings of *SlERF.F5*-RNAi lines showed more sensitive characteristics to ACC and MeJA (Supplementary Fig. S1,2). In summary, suppression of *SlERF.F5* gene can promote the leaf senescence by affecting the expression levels of genes in the ethylene biosynthesis pathway, the jasmonic acid signal transduction pathway, and downstream transcription factors of the JA receptor.

According to previous reports, *AtERF2* positively regulates jasmonic acid-sensitive defense gene expression, while *AtERF4* negatively regulates jasmine-sensitive defense gene expression (McGrath et al. 2005). Given the role of ERF and jasmonic acid-related genes, we choose SlMYC2 and SlERF.F5 for yeast two-hybrid experiments. The results showed that SlERF.F5 could interact with SlMYC2. Studies have shown that increased expression of *SlMYC2* gene will promote leaf senescence, which indicated that *SlMYC2* plays a positive role in leaf senescence. The experimental results proved that *SlERF.F5* plays a negative regulatory role in leaf senescence, and its expression gradually decreases during leaf senescence. In the *SlERF.F5*-RNAi lines, the expression level of *SlMYC2* was higher than that of WT. The above results indicated that silencing of *SlERF.F5* gene might relieve its inhibition to *SlMYC2*, thereby promoting leaf senescence.

Combining previous studies and our experimental results, we propose a working model that describes how *SlERF.F5* and *SlMYC2* regulate leaf senescence under the influence of hormones and environmental stress (Fig. 10). Ethylene transcription factor *SlERF.F5* is an essential negative regulator of leaf senescence. The expression of *SlSAG12* increased in the leaves of the *SlERF.F5*-RNAi lines, indicating that inhibition of *SlERF.F5* could induce the expression of the *SlSAG12* gene to promote leaf senescence.

Under the condition of age or darkness, inhibits the expression of *SlERF.F5*, the expression levels of *CHLH*, *CHLM*, *POR*, *CAO1*, *GUN4*, *PPH*, *SGR1*, *RBCS PPH*, *SGR1*, *RBCS*, *ACREA* and *LHCA1* were reduced, thereby accelerating leaf senescence. On the one hand, it inhibits *SlERF.F5* will induce the expression of *SlMYC2*, and the two interact to promote leaf senescence in silent lines. On the other hand, *SlMYC2* could directly activate *SGR1* and other chlorophyll catabolic enzyme genes during the leaf senescence induced by JA (Zhu et al. 2015). Besides, *SlERF.F5* can induce the expression of some JAZ proteins in the jasmonic acid signal transduction pathway (JAZ1, JAZ2, JAZ4, JAZ7, JAZ11) among which JAZ7 can interact with COI1 and, or SlMYC2 to regulate leaf senescence induced by darkness (Yu et al. 2016). However, the interaction between JAZ7 and WRKY57 to promote leaf senescence was studied in Arabidopsis thaliana, and its function in tomatoes has not yet been reported. If the regulatory mechanism model was to be perfected, further experimental verification was needed. Overall, the physiological and molecular mechanism analysis showed that *SlERF.F5* plays a vital role in regulating the leaf senescence induced by darkness. Ethylene and jasmonic acid play a synergistic role in regulating leaf senescence.

Declarations

Author contribution statement

G. C, S. Z, and Z. H designed and managed the research work and improved the manuscript. Y. C, P. F, B. T, Q. X performed the experiments. Y. C wrote the manuscript. All authors read and approved the manuscript.

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Conflict of interest

All authors have read and approved this version of the article, and due care has been taken to ensure the integrity of this work. The authors declare that they have no conflict of interest.

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Figures

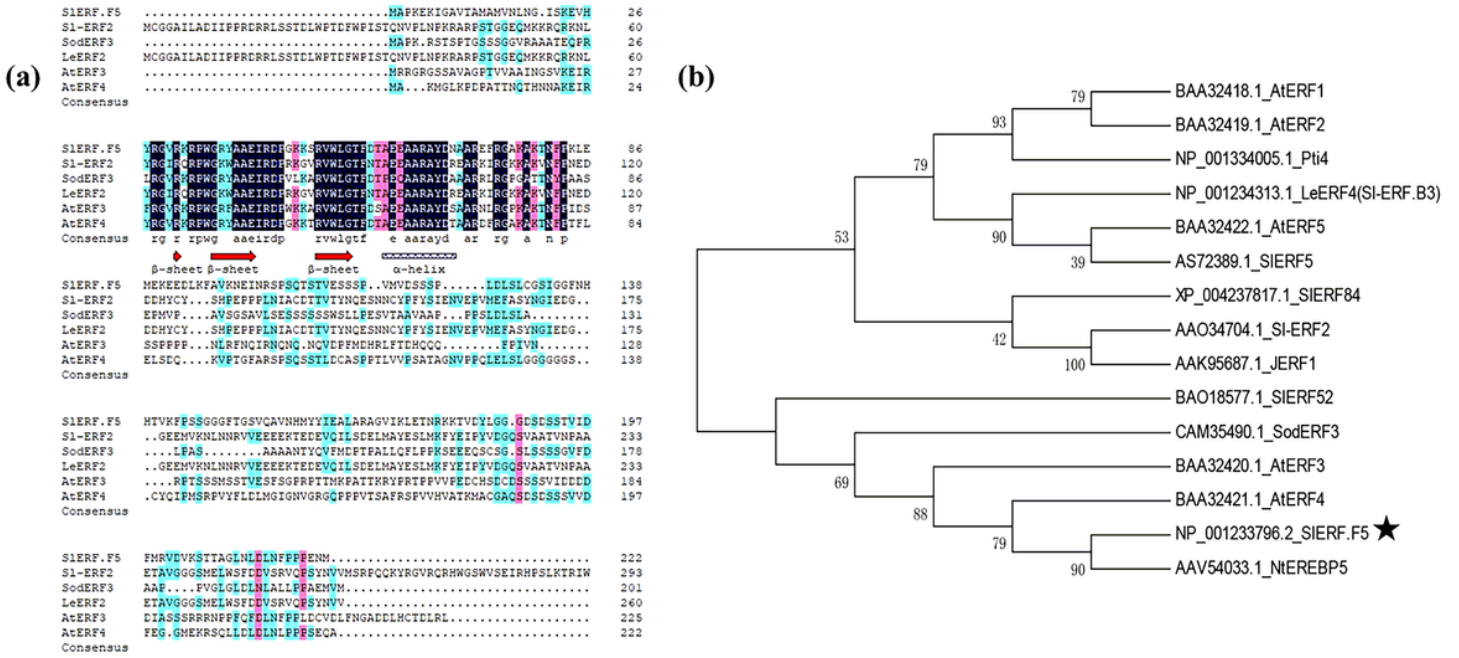

Figure 1

Sequence and expression analysis of SlERF.F5 (a) Protein sequence alignment of SlERF.F5 and other ethylene transcription factors of ERF family. The same amino acid is indicated in black, and its protein sequence has three β-sheets and an α-helix. (b) Phylogenetic analysis of SlERF.F5 and other ERF proteins was constructed using MEGA 5.0 software and the Neighbor-Joining method, bootstrap analysis of 1000 replicates. The accession numbers for other proteins are as follows: AtERF1 (BAA32418.1), AtERF2 (BAA32419.1), Pti4 (NP_001334005.1), LeERF4 (SI-ERF.B3) (NP_001234313.1), AtERF5 (BAA32422.1), SlERF5 (AS72389.1), SlERF84 (XP_004237817.1), SI-ERF2 (AAO34704.1), JERF1 (AAK95687.1), SodERF3 (CAM35490.1), AtERF3 (BAA32420.1), AtERF4 (BAA32421.1), SlERF.F5 (NP_001233796.2), NtEREBP5 (AAV54033.1), SlERF52 (BAO18577.1).represent the genes studied in this article.

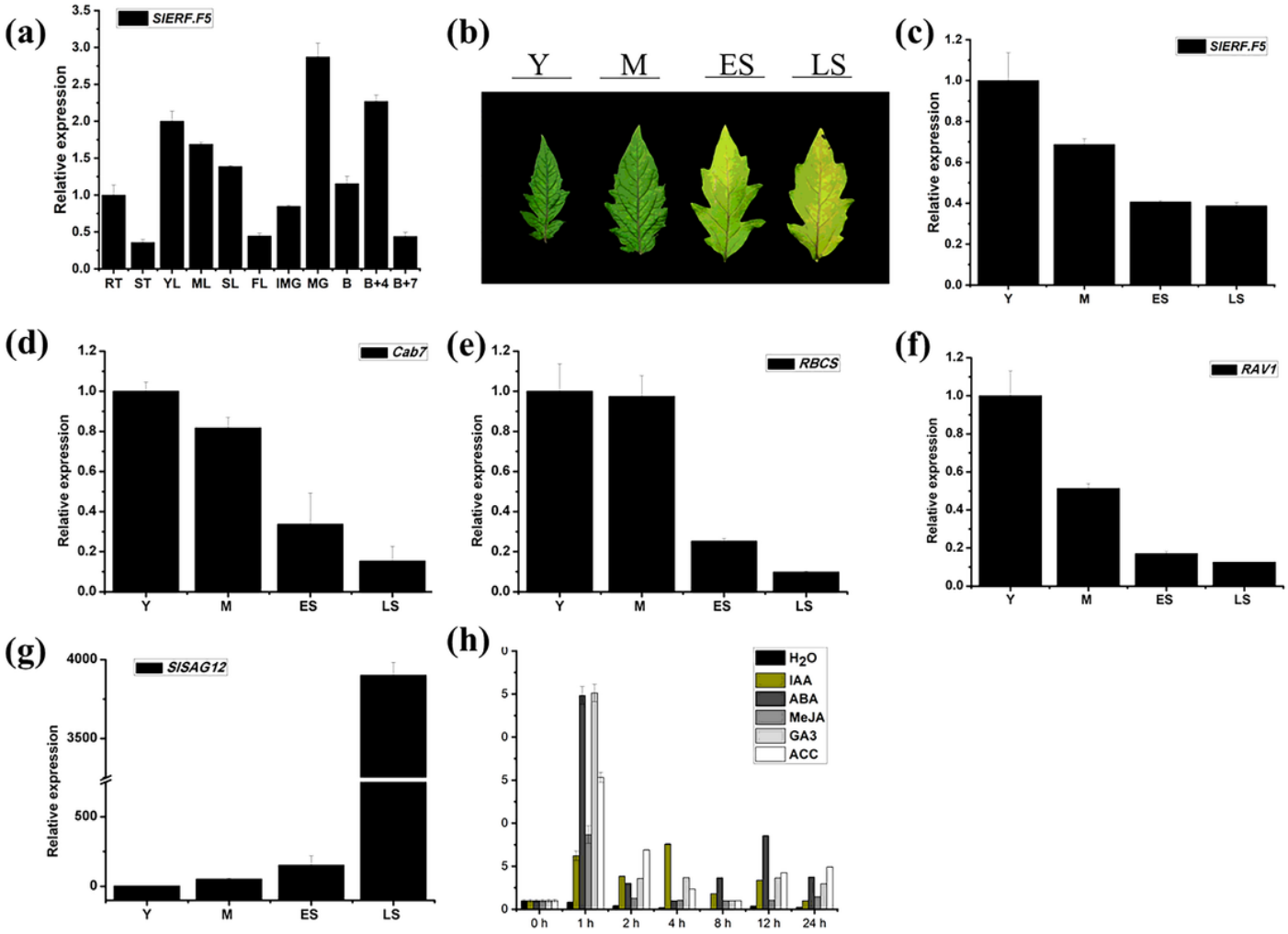

Figure 2

Expression pattern of SlERF.F5 (a) The expression level of SlERF.F5 in various tissues of WT. The expression level of SlERF.F5 in various tissues of WT. RT (root), ST (stem), YL (young leaf), ML (mature leaf), SL (senescence leaf), FL (flower), IMG (immature fruit), MG (green fruit), B (breaker fruit), B+4 (4 days after breaker fruit), B+7 (7 days after breaker fruit). (b) Different development stages of tomato leaves, Y (young tomato leaves), M (mature leaves), EL (early senescence leaves, yellowing area > 25%) and LS (late senescence leaves, yellowing area > 50%). (c-g) qRT-PCR analysis of the expression levels of SlERF.F5, Cab7, RBCS, Rav7, and SlSAG12 in wild-type tomato leaves at different developmental stages. The experimental results were repeated three times in biology. (h) qRT-PCR analysis of the expression patterns of SlERF.F5 in response to IAA, ABA, MeJA, GA3, and ACC. All experimental data are the mean ± SD of three independent experiments. Error bars indicate SE.

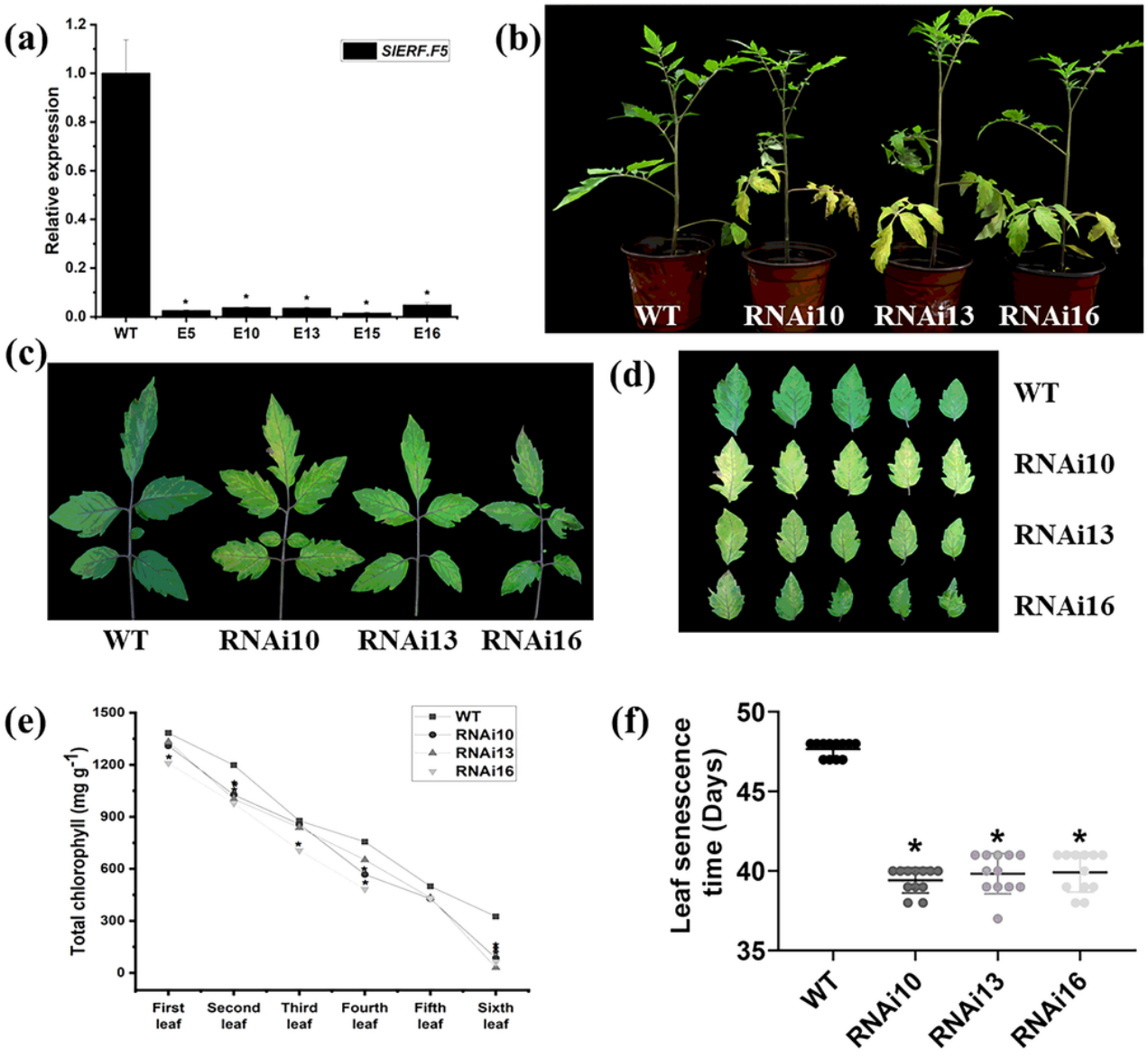

Figure 3

Silencing of SlERF.F5 causes premature senescence of tomato leaves (a) The expression level of SlERF.F5 in mature leaves of WT and SlERF.F5-RNAi lines. (b) The senescence phenotype of 10 weeks old WT, RNAi10, RNAi13, and RNAi16 lines. (c) The senescence phenotype of the fifth leaf of WT, RNAi10, RNAi13, and RNAi16 lines. (d) Isolation of leaves from 12-week-old WT and RNAi10, RNAi13, RNAi16 lines. (e) Chlorophyll content of each leaf in WT, RNAi10, RNAi13, and RNAi16 lines. (f) Leaf senescence time of WT, RNAi10, RNAi13 and RNAi16 lines. Three biological replicates were performed for each content determination. The data represent the mean from three replicates with three biological repeats. *, indicate $P < 0.05$, between the wild type and others by t-test. Error bars indicate SE.

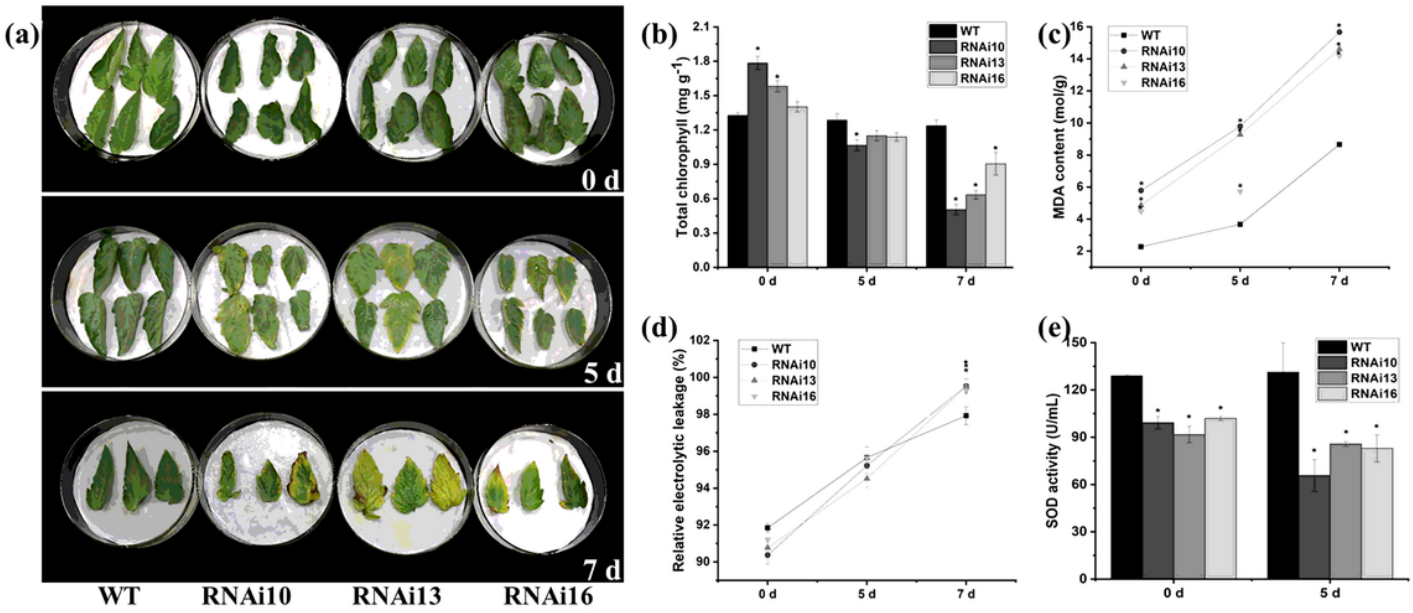

Figure 4

Silencing of SlERF.F5 promotes dark-induced leaf senescence (a) The leaves of the WT and SlERF.F5-RNAi lines (RNAi10, RNAi13, RNAi16) were treated in the dark for 0 days, 5 days, and 7 days. (b) Leaf chlorophyll content of WT and RNAi10, RNAi13, RNAi16 lines at 0, 5, and 7 days in the dark. (c) The MDA (malonaldehyde) content of the leaves of the WT and RNAi10, RNAi13, RNAi6 lines at 0, 5, and 7 days of dark treatment. (d) Relative conductivity of leaves of WT and RNAi10, RNAi13, and RNAi16 lines in dark treatment for 0 days, 5 days, 7 days. (e) SOD activity in leaves of WT and RNAi10, RNAi13, and RNAi16 lines at 0 and 5 days after dark treatment. The data represent the mean from three replicates with three biological repeats. *, indicate $P < 0.05$, between the wild type and others by t-test. Error bars indicate SE.

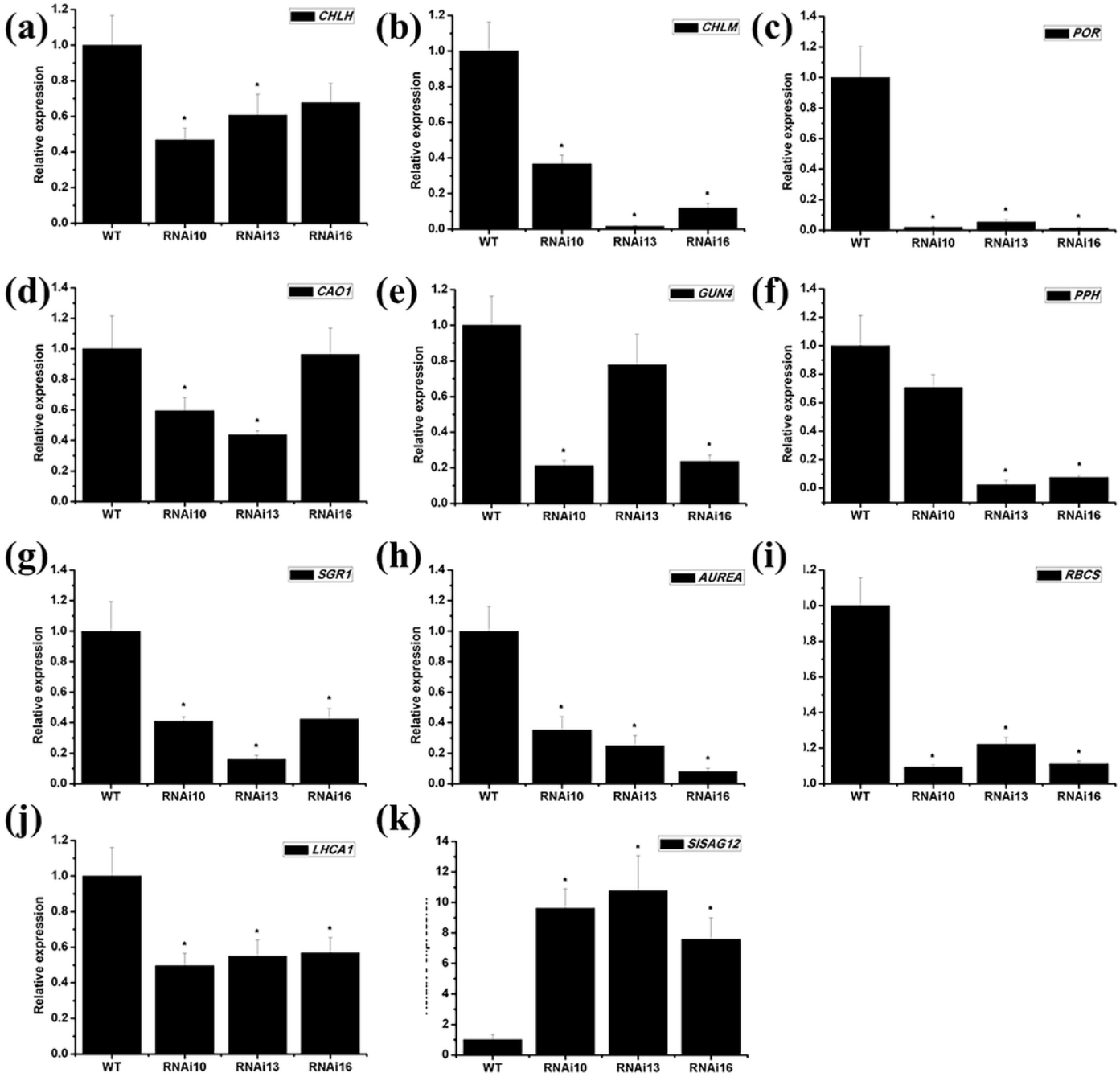

Figure 5

Silencing of SlERF.F5 altered the expression of genes involved chloroplast development, chlorophyll metabolism, and senescence markers (a-e) qRT-PCR analysis of CHLH, CHLM, POR, CAO1, GUN4 (chloroplast development-related genes) expression levels in WT and RNAi10, RNAi13, RNAi16 lines. (f-i) qRT-PCR analysis of chloroplast metabolism related genes PPH, SGR1, RBCS, AUREA expression levels in WT and RNAi10, RNAi13, RNAi16 lines. (j) Quantitative RT-PCR analysis of the expression level of light-responsive gene LHCA1 in WT and RNAi10, RNAi13, RNAi16 lines. (k) qRT-PCR analysis of the expression level of senescence marker gene SlSAG12 in WT and RNAi10, RNAi13, RNAi16 lines. The data represent

the mean from three replicates with three biological repeats. *, indicate P < 0.05, between the wild type and others by t-test. Error bars indicate SE.

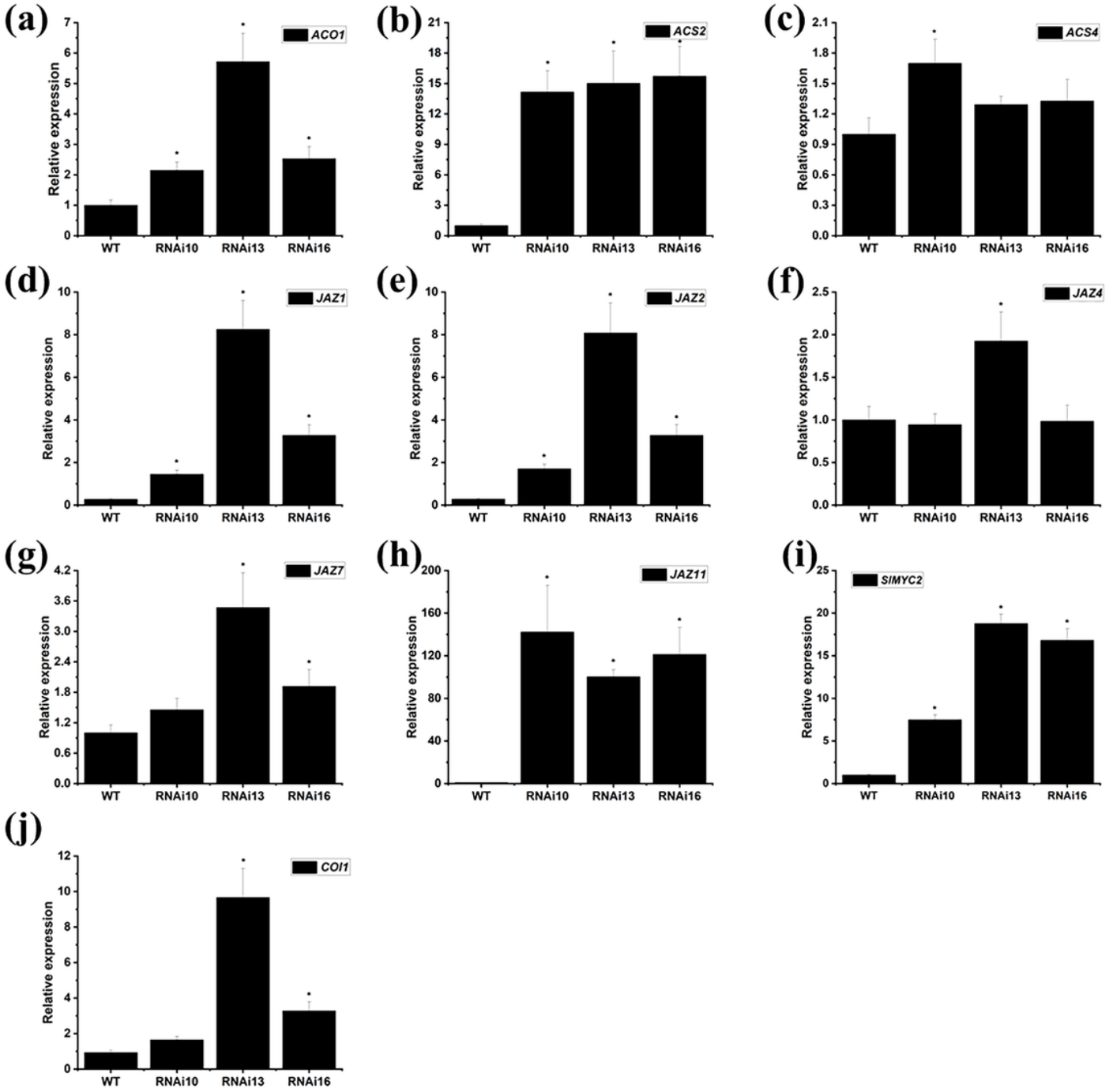

Figure 6

Silencing of SlERF.F5 affects the expression of ethylene, and jasmonic acid signaling pathway genes (a-c) qRT-PCR analysis of the expression levels of ethylene biosynthetic pathway genes ACO1, ACS2 and ACS4 in WT and RNAi10, RNAi13, RANi16 lines. (d-h) Quantitative RT-PCR analysis of jasmonic acid signal transduction pathway genes JAZ1, JAZ2, JAZ4, JAZ7, and JAZ11 expression levels in WT and RNAi10, RNAi13, RANi16 lines. (i-j) qRT-PCR analysis of the expression levels of jasmonic acid receptor

downstream transcription factor MYC2 and jasmonic acid-dependent transcription factor COI1 in WT and RNAi10, RNAi13, RNAi16 lines. The data represent the mean from three replicates with three biological repeats. *, indicate P < 0.05, between the wild type and others by t-test. Error bars indicate SE.

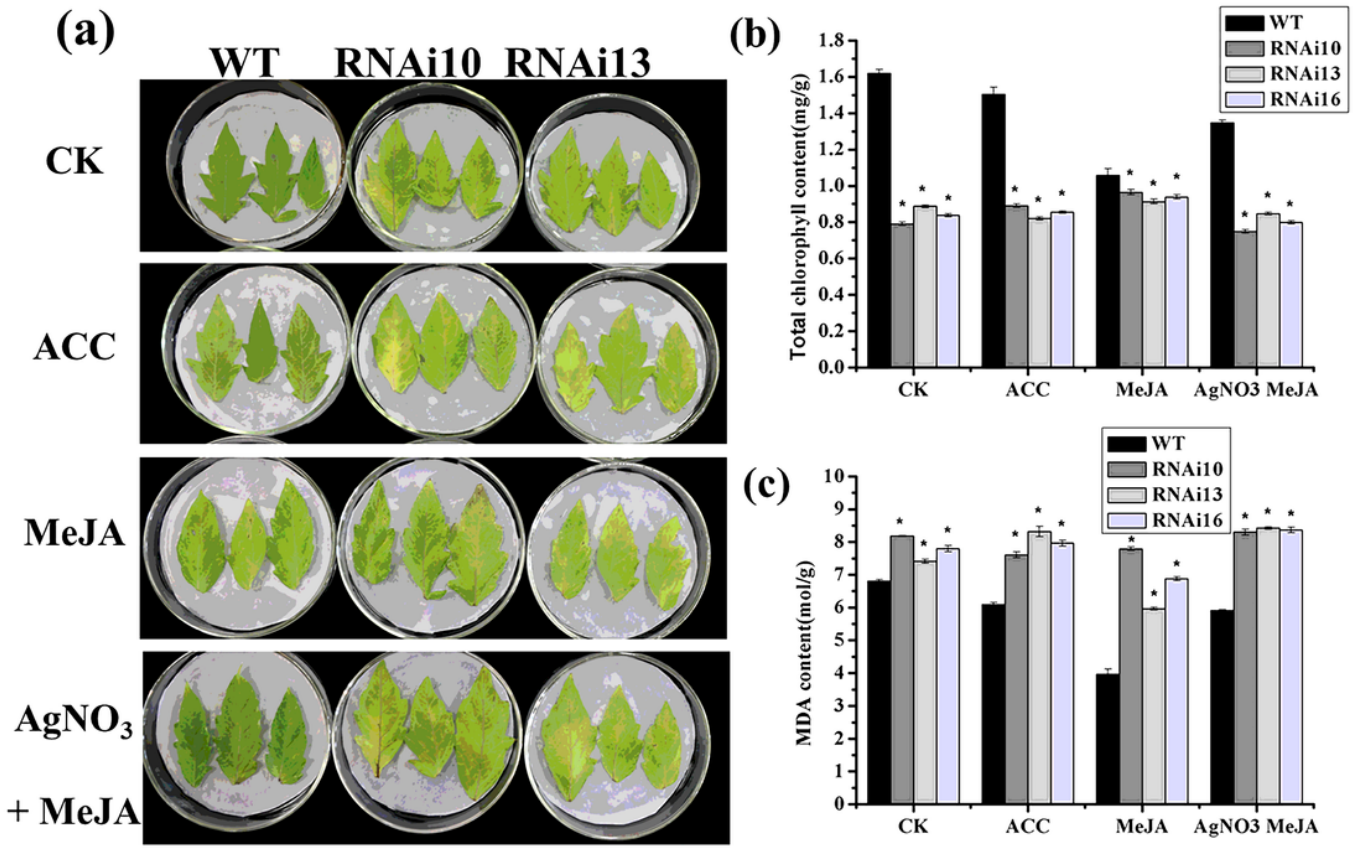

Figure 7

Silencing of SlERF.F5 promotes dark, ethylene and JA induced leaf senescence (a) Senescence phenotypes of isolated leaves of WT, RNAi10, RNAi13, RNAi16 lines treated with ACC, MeJA, and AgNO3+MeJA in the dark. Under dark conditions, the isolated leaves were treated with water (control), 100 μM ACC, or 50 μM MeJA for seven days. For AgNO3+MeJA treatment, the leaves were pretreated with 10 μM AgNO3 for 1 h, washed with water, and then treated with 50 μM MeJA in the dark for seven d. (b) The measurement of chlorophyll content comes from hormone-treated isolated leaves. (c) MDA content was measured from hormone-treated leaves. The data represent the mean from three replicates with three biological repeats. *, indicate P < 0.05, between the wild type and others by t-test. Error bars indicate SE.

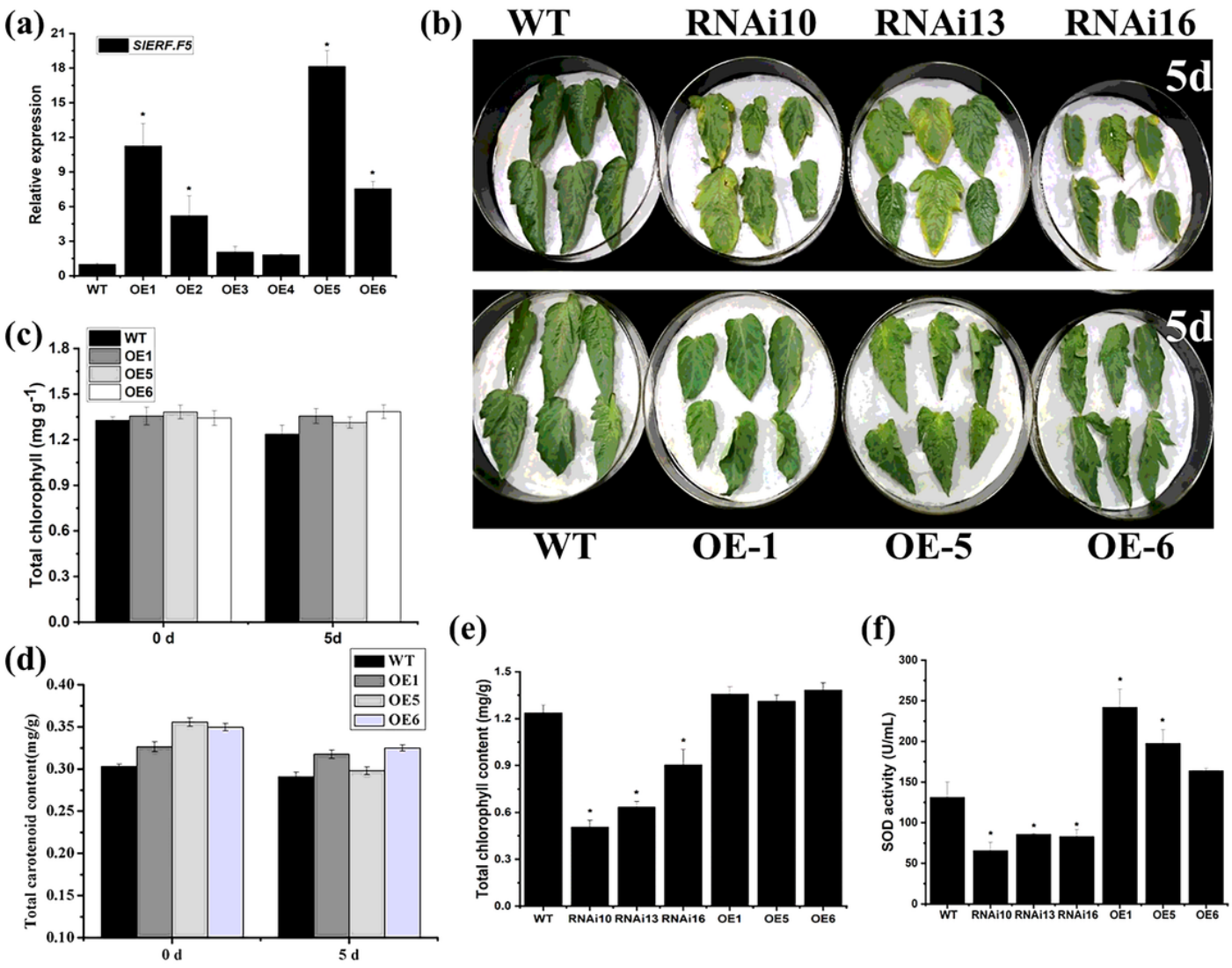

Figure 8

Overexpression of SlERF.F5 does not promote leaf senescence (a) The expression level of SlERF.F5 in mature leaves of WT and SlERF.F5-OE lines. (b) Dark-induced leaf senescence for five days in WT and RNAi10, RNAi13, RNAi16 lines and OE-1, OE-5, OE-6 lines. (c) Analysis of chlorophyll content in WT and OE-1, OE-5, OE-6 lines. (d) Analysis of carotenoid content in WT and OE-1, OE-5, OE-6 lines. (e) Analysis of chlorophyll content in WT and RNAi10, RNAi13, RNAi16 lines and OE-1, OE-5, OE-6 lines. (f) Analysis of SOD activity in WT and RNAi10, RNAi13, RNAi16 lines and OE-1, OE-5, OE-6 lines. The data represent the mean from three replicates with three biological repeats. *, indicate $P < 0.05$, between the wild type and others by t-test. Error bars indicate SE.

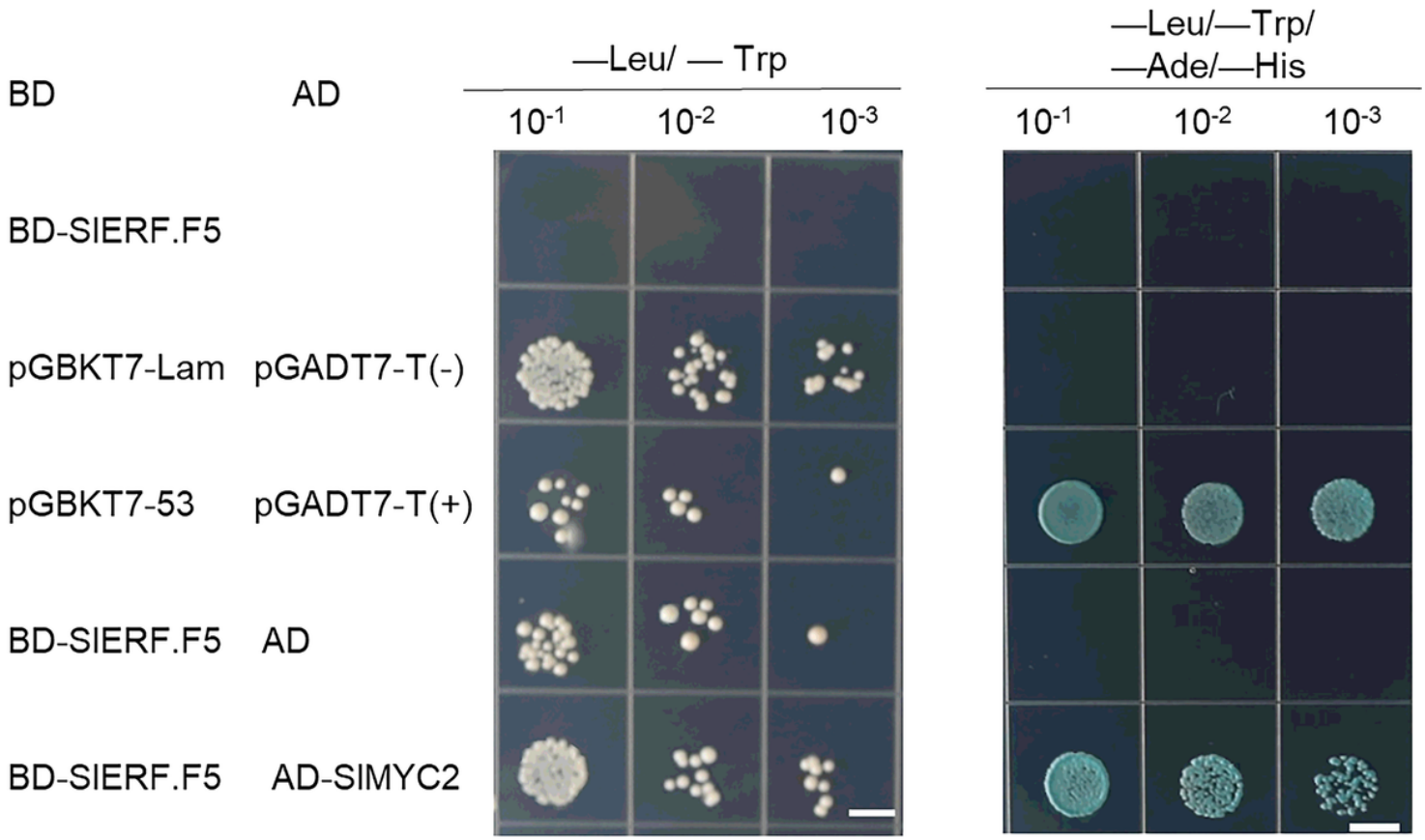

Figure 9

SlERF.F5 Physically Interacts with SlMYC2 Yeast two-hybrid experiment indicated that SlERF.F5 interacted with SlMYC2. Co-transformation of pGADT7-T and pGBKT7-53 as a positive control; co-transformation of pGADT7-T and pGBKT7-Lam as a negative control; single-transformation of BD-SlERF.F5 and co-transformation with AD to verify self-activation; co-transformation of BD-SlERF.F5 and AD-SlMYC2 as an experimental group. The whitewire frame represents 1 cm.

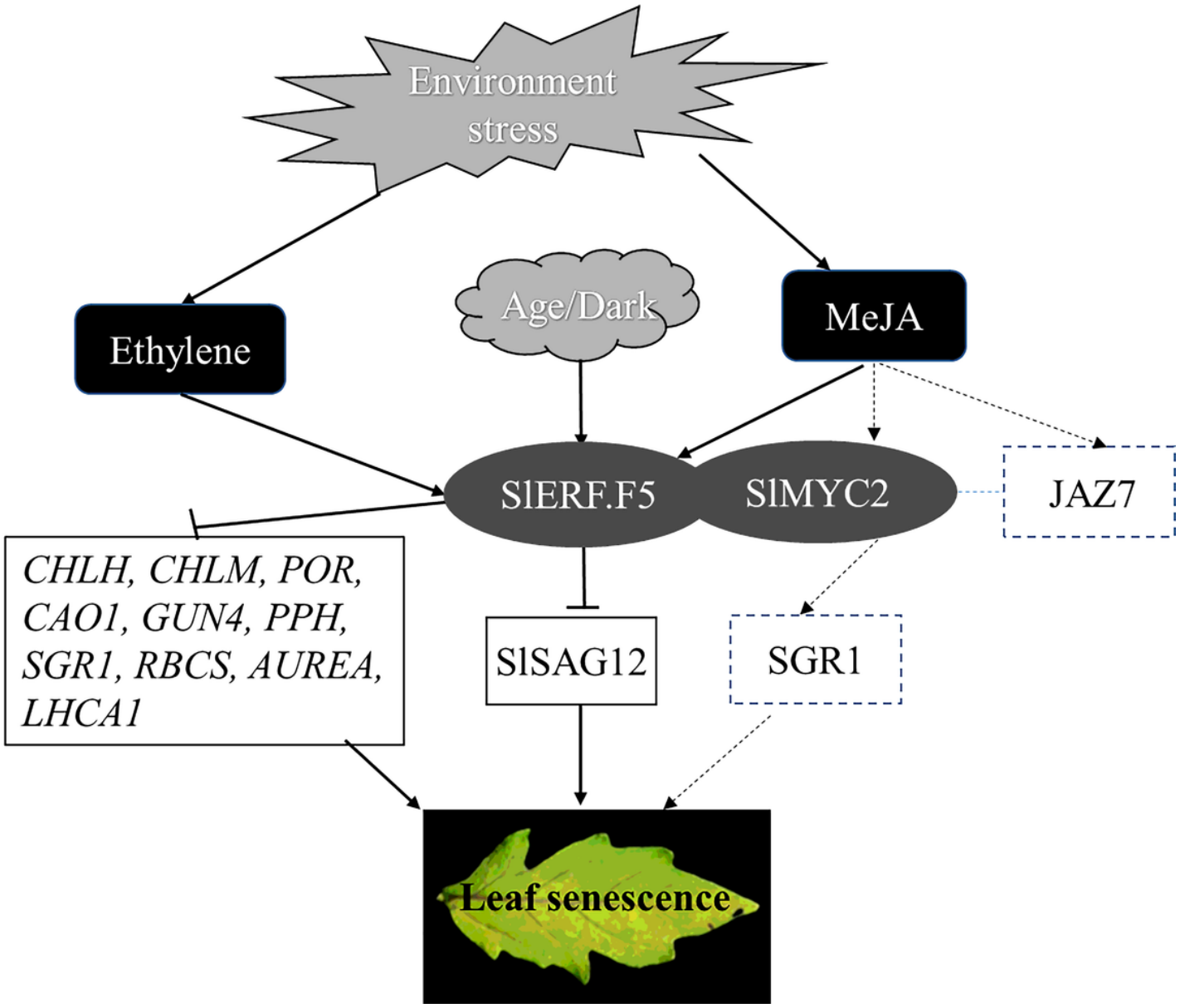

Figure 10

A proposed model illustrates the cascade regulation of SlERF.F5-SlMYC2 in leaf senescence

Supplementary Files

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