The histone H3K27 demethylase SlJMJ4 promotes dark- and ABA-induced leaf senescence in tomato

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Abstract
Leaf senescence is a highly-programmed developmental process during the plant life cycle. ABA plays an important role in leaf senescence. However, the mechanism underlying ABA-mediated leaf senescence, particularly the upstream epigenetic regulatory network, remains largely unclear. Here, we demonstrated that SlJMJ4, a Jumonji C (jmjC) domain-containing protein in tomato (Solanum lycopersicum), specifically demethylates di- and trimethylations of lysine 27 of histone H3 (H3K27) in vitro and in vivo. Overexpression of SlJMJ4 results in a premature senescence phenotype and promotes dark- and ABA-induced leaf senescence in tomato. Under dark conditions, SlJ MJ4-promoted leaf senescence is associated with upregulated expression of transcription factors (SlORE1 and SlNAP2) and senescence-associated genes (SlSAG113 and SlSAG12) via removal of H3K27me3. In response to ABA, overexpression of SlJMJ4 increases its binding at the loci of SlORE1, SlNAP2, SlSAG113, SlSAG12, SlABI5, and SlSNCED3 and decreases their H3K27me3 levels, thereby activating their expression and mediating ABA-induced leaf senescence in tomato. Taken together, these results demonstrate that SlJMJ4 plays a positive role in leaf senescence in tomato and functions in ABA-induced leaf senescence by binding to many key genes related to ABA synthesis and signaling, transcription regulation, and senescence, thus promoting their H3K27me3 demethylation.

Introduction
Senescence is the last stage in the plant life cycle. Plant senescence results in cell, tissue, organ, or even organism death [1]. Leaf senescence is characterized by chlorophyll degradation, reduced photosynthesis, and nutrient remobilization, which is critical for crop fitness and productivity. Leaf photosynthesis is essential for maximizing the carbohydrate level in seeds or fruit, and delaying senescence therefore facilitates increases in yield. In addition, efficient senescence is beneficial for maximizing stored nutrients [2]. A better understanding of the regulatory mechanism of leaf senescence has high economic relevance for decreasing yield losses.

Leaf senescence is a complicated programmed process controlled by environmental and endogenous signals that involves several layers of regulation, such as transcriptional/post-transcriptional regulation, translational/post-translational regulation, and epigenetic regulation [3]. Transcriptional regulation has crucial roles in leaf senescence. Transcription factors from the NAC [4] and WRKY [5] families have emerged as important regulators of leaf senescence in Arabidopsis and rice. Other transcription factors, such as the MYB [6], bHLH [7], bZIP [8], and AP2/EREBP [9] families, are also implicated in the regulation of leaf senescence. These transcription factors constitute complex regulatory networks with hormone signaling pathways that control the expression of senescence-associated genes (SAGs), thus regulating leaf senescence [10]. In recent years, great attention has been paid to the functions of epigenetic modifications, such as DNA methylation, chromatin remodeling, and histone modification, in the regulation of leaf senescence [11–14]. Yuan et al. [11] reported that DEMETER-like protein 3 (DML3) activates several SAGs by DNA demethylation in Arabidopsis, thereby regulating leaf longevity. Chen et al. [12] found that histone deacetylase HDA9 interacts with WRKY53 to promote the onset of leaf senescence. Cho et al. [13] revealed that loss of DRD1 and DDM1, two SWI2/SNF2-like chromatin-remodeling proteins, postponed Arabidopsis leaf senescence. In addition, REF6 promotes the recruitment of BRM (an SWI2/SNF2-type ATPase) to target numerous SAGs [14]. Therefore, different epigenetic mechanisms are implicated in leaf senescence modulation.
Histone methylation, an important epigenetic marker, plays roles in different biological processes, such as maintenance of genome integrity and transcriptional regulation. It is written via histone methyltransferases and eliminated by histone demethylases [15]. In general, histone lysine demethylases can be classified into two types, Jumonji C (JmjC) domain-containing proteins (JMJs) and lysine-specific demethylase 1 (LSD1). LSD1 family proteins act only on mono- and dimethylated lysine, whereas JMJs show demethylase activity towards mono-, di-, and trimethylated lysine. JMJs represent the majority of histone lysine demethylases and catalyze the demethylation of lysine via oxidation, with α-ketoglutarate and Fe (II) iron as required cofactors. JMJs have been reported to regulate flowering [16], circadian rhythm [17], fruit ripening [18], stress response [19], and seed germination [20]. Recently, two important studies revealed that JMJs are involved in the regulation of Arabidopsis leaf senescence [21, 22]. REF6/JMJD12, an H3K27 demethylase, accelerates the premature activation of leaf senescence by binding to regulators of NON-YELLOWING 1 (NYE1) and directly upregulating the transcription of chlorophyll degradation genes and SAGs [21]. JMJD16, a specific H3K4 demethylase, negatively regulates leaf senescence in Arabidopsis by reducing H3K4me3 at WRKY53 and SAG201 loci [22]. Based on these studies, leaf senescence is closely associated with histone modification. However, it remains unclear how histone modifications regulate leaf senescence in other plants.

Tomato, an economically important horticultural crop, is used as a model system to investigate the growth and ripening of fleshy fruit. However, there is little research on the regulation of leaf senescence in tomato. Previous studies have shown that leaf senescence in tomato is accompanied by decreased H3K27me3 levels and increased SAG expression [23], implying that H3K27me3 demethylases may be involved in the regulation of tomato leaf senescence. Here, we demonstrated that SlJMJD4 is an H3K27 demethylase that positively regulates leaf senescence. Overexpression of SlJMJD4 reduces H3K27me3 levels in relation to the upregulated expression of SAGs and genes related to ABA synthesis and signaling. These results illustrate a new possible mechanism by which the H3K27 demethylase SlJMJD4 is involved in dark- and ABA-induced leaf senescence in tomato.

Results

Expression of JMj genes in young and senescent leaves of tomato

In tomato, the JMj family has 20 members, which can be categorized into five subfamilies, KDM4/HDM3 (SlJMJD1, SlJMJD2, SlJMJD3, SlJMJD4), KDM5/JAR2D1 (SlJMJD5, SlJMJD6, SlJMJD7, SlJMJD8), JMD6 (SlJMJD9, SlJMJD10, SlJMJD11), KDM3/HDM2 (SlJMJD12, SlJMJD13, SlJMJD14, SlJMJD15, SlJMJD16, SlJMJD17), and JMJC/DOG (SlJMJD1, SlJMJD2, SlJMJD3) [18]. We compared the expression levels of all 20 SlJMJs in young and senescent leaves and found that several SlJM genes, including SlJMJD4, SlJMJD13, SlJMJD15, SlJMJD1, and SlJMJD2, were significantly upregulated in senescent leaves compared with young leaves. Among these genes, SlJMJD4 showed the highest degree of upregulation (Fig. 1). These results imply that SlJMJD4 may have an important function in leaf senescence. Therefore, we chose to further elucidate the possible role of SlJMJD4 in regulating tomato leaf senescence.

Bioinformatic analysis and subcellular localization of the SlJMJD4 protein

Sequence analyses showed that SlJMJD4 harbors a zf-C5HC2 domain, a highly conserved JmjC domain, and a JmjN domain (Fig. S1a). Based on the KDM4 subfamily sequences of its human, Arabidopsis, and rice homologs, we determined that SlJMJD4 had high homology to AtJMJD13 and OsJMJD706 (Fig. S1b). AtJMJD13 is an H3K27me3 demethylase and a photoperiod- and temperature-dependent flowering inhibitor [24], whereas OsJMJD706 has H3K9me2/3 demethylase activity and is implicated in the regulation of rice flowering [25]. Sequence alignment of homologous proteins revealed that tomato JMJs contain conserved Fe (II)- and α-KG-binding amino acids within the cofactor binding site (Fig. S1c), which are required for catalyzing demethylation via a hydroxylation reaction [18]. Subcellular localization analysis showed that SlJMJD4 was located in the nucleus (Fig. S1d), consistent with a possible role in the regulation of chromatin structure. Together, these results imply that SlJMJD4 may be capable of H3K27 or H3H9 demethylation and may play a positive role in gene activation.

SlJMJD4 has specific H3K27me3/2 demethylase activity in vivo and in vitro

To confirm the site of SlJMJD4 action, we analyzed the histone demethylase activity of SlJMJD4 in vivo and in vitro. For in vivo analysis, we compared the histone methylation profiles of WT and SlJMJD4-OE plants using two independent lines, SlJMJD4-OE43 and SlJMJD4-OE50. Western blotting analysis showed that the di- and trimethylation levels at H3K27 were clearly lower in SlJMJD4-OE43 and SlJMJD4-OE50 plants than in the WT (Fig. 2a). However, no differences were observed in the levels of H3K27me1, H3K9me1/2/3, H3K4me1/2/3, or H3K36 me1/2/3 between the WT and SlJMJD4-OE plants. For in vitro analysis, calf thymus type I-A histone was used as the substrate, and Fe(NH4)2(SO4)2, α-ketoglutarate, and ascorbate were used as the coenzymes of the enzymatic reaction. As shown in Fig. 2b, SlJMJD4-GST recombinant protein reduced H3K27me2/3 levels, yet it did not affect the levels of H3K27me1, H3K9me1/2/3, H3K4me1/2/3, or H3K36me1/2/3 in vitro. Taken together, these results revealed that SlJMJD4 is a specific H3K27me2/3 demethylase in tomato.
Figure 1. Expression of SLJMJ genes in young and senescent tomato leaves from 2-month-old plants. Young leaves were obtained from the top part of the stem, and senescent leaves were obtained from the bottom part of the stem. ACTIN was used as the reference gene. The values are shown as the mean ± SE of three biological replicates (Student’s t-test, **P < 0.01).

SlJMJ4 is related to leaf senescence in tomato

To explore the function of SlJMJ4 in regulating leaf senescence, we generated SlJMJ4-overexpressing lines in the Ailsa Craig background. Eighteen independent transgenic lines were obtained, and two stable lines expressing high levels of SlJMJ4, SlJMJ4-OE43 and SlJMJ4-OE50, were selected for further analysis (Fig. 3a, b). Phenotypic analysis showed that SlJMJ4-overexpressing plants exhibited obvious premature senescence (Fig. 3a, b). The yellow ratios were significantly higher (Fig. 3c) and the chlorophyll contents were much lower in leaves of 12-week-old SlJMJ4-OE plants than in those of the WT (Fig. 3d).

In addition, transcription levels of SlJMJ4 were analyzed in different tissues, including roots, flowers, young leaves, senescent leaves, and fruits at various ripening stages, as well as under dark conditions. The results showed that SlJMJ4 was slightly expressed in young leaves, flowers, and roots, moderately expressed in unripe and ripe fruits, and highly expressed in senescent leaves and fruits (Fig. 3e). It appears that the expression of SlJMJ4 is associated with tissue senescence. Moreover, dark treatment induced the expression of SlJMJ4 in WT leaves (Fig. 3f), and the ABA content was higher in SlJMJ4-overexpressing leaves than in WT leaves under dark induction (Fig. 3g). Collectively, these data suggest that SlJMJ4 plays a positive role in leaf senescence of tomato, which may be related to the phytohormone ABA.

SlJMJ4 accelerates dark-induced leaf senescence in tomato

Dark treatment is known to induce senescence of detached leaves in plants, making it a good model for
studying leaf senescence [26]. Here, we examined the possible role of SlJMJ4 in regulating dark-induced leaf senescence in tomato. The detached leaves of the SlJMJ4-OE43 and SlJMJ4-OE50 lines showed strong senescence phenotypes after 12 d of dark incubation (Fig. 4a), with significantly lower chlorophyll content (Fig. 4b) and higher ion leakage (Fig. 4c) compared with the WT. $Fv/Fm$ is an important parameter for evaluating leaf senescence. During dark incubation, the $Fv/Fm$ value of detached WT leaves gradually decreased. However, the value decreased dramatically in SlJMJ4-OE43 and SlJMJ4-OE50 lines (Fig. 4d), indicating that SlJMJ4 promoted the senescence of detached leaves under dark conditions. Moreover, the chlorophyll fluorescence images were consistent with the $Fv/Fm$ values in WT and SlJMJ4-OE lines during dark incubation (Fig. 4e). Chlorophyll degradation genes, senescence-associated genes, and transcription factor genes such as NACs and WRKYs are considered to be important positive regulators of leaf senescence. We examined the expression of many genes related to senescence regulation, including SlSAG12/13/15/101/113, SlEIN3, SlNOR, SlORE1, SlNAP2, and SlWRKY53. As shown in Fig. 4f, the expression levels of these genes were markedly upregulated in the SlJMJ4-OE43 line compared with the WT under dark conditions.

**SlJMJ4 upregulates the expression of SlSAG12, SlSAG113, SlNAP2, and SlORE1 by removing H3K27me3 under dark conditions**

To further investigate the role of SlJMJ4 in leaf senescence, we performed a ChIP-seq analysis to identify the direct targets of SlJMJ4 in the tomato leaves. Leaves from 2-month-old SlJMJ4-OE plants were used for immunoprecipitation with anti-GFP antibody and anti-IgG antibody (negative control). A total of 4097 genes, corresponding to 5004 common binding peaks from three biological replicates, were identified [Supplementary Table S1; Supplementary Fig. S2a] and were distributed in different genomic regions, including introns, exons, transcription start sites, promoters, and intergenic areas [Supplementary Fig. S2b]. The predominant DNA-binding sites of SlJMJ4 were distributed in intergenic areas and exons [Supplementary Fig. S2b]. Meta-gene analysis also showed that SlJMJ4 binding sites were most significantly enriched at transcription end sites [Supplementary Fig. S2c]. Motif enrichment analysis showed that the most predominant motif in SlJMJ4-binding sequences was YACGTY (where Y represents A, T, G, or C), which is a binding site of bZIP transcription factors, including HY5, ABI5, TGA4, TGA6, and JUND [Supplementary Fig. S2d; Supplementary Table S2]. This result implies that SlJMJ4 may be recruited by bZIP transcription factors to regulate gene expression. GO and KEGG pathway enrichment analyses showed that these SlJMJ4-targeted genes were associated with photosynthesis, light harvesting, carboxylic acid metabolism, stress response, and programmed cell death [Supplementary Figs. S3a and b].

The trimethylation of H3K27 is a global epigenetic mark that is usually associated with gene repression [27]. As SlJMJ4 has H3K27me3 demethylase activity, we next examined whether SlJMJ4 regulates the expression of genes related to senescence regulation by H3K27me3 demethylation during dark-induced leaf senescence. We selected four genes related to senescence regulation, SlSAG12, SlSAG113, SlNAP2, and SlORE1 [Fig. 5a–d], and we compared their H3K27me3 levels in WT and SlJMJ4-OE43 leaves at 12 d under dark conditions by ChIP-qPCR with an anti-H3K27me3 antibody. As shown in Fig. S5a–h, SlSAG12, SlSAG113, SlNAP2, and SlORE1 showed no differences in H3K27me3 levels between SlJMJ4-OE43 and WT lines in the control treatment (continuous light). However, these genes displayed significantly reduced levels...
Figure 3. SlJMJ4 is implicated in the regulation of leaf senescence in tomato. a Phenotype of SlJMJ4-OE transgenic plants. Twelve-week-old plants from WT and SlJMJ4-OE lines are displayed. b The expression of SlJMJ4 in SlJMJ4-OE43 and SlJMJ4-OE50 compared with the WT. Expression was analyzed in one-month-old seedlings. ACTIN was used as the reference gene. c Yellow leaf ratio of 12-week-old WT, SlJMJ4-OE43, and SlJMJ4-OE50 plants. Leaves with >50% yellowing were counted and divided by the total number of leaves. d Chlorophyll content of the bottom third leaf on stems of 8-week-old (8 W), 10-week-old (10 W), 12-week-old (12 W), and 14-week-old (14 W) WT, SlJMJ4-OE43, and SlJMJ4-OE50 plants. Chlorophyll content was measured with a SPAD meter. e SlJMJ4 transcript abundance in different tissues of WT tomato plants (cv. Ailsa Craig). IMG, immature green; MG, mature green; Breaker, color break; Orange, turning orange; Red, mature red. f Expression of SlJMJ4 in young detached leaves from the top of the stems of 2-month-old WT plants at 0 d or 12 d after dark incubation. Leaves under daylight conditions were used as the controls. g ABA content in leaves from 2-month-old WT and SlJMJ4-OE plants at 12 d after dark incubation. In b, c, d, f, and g, the values are shown as the mean ± SE of three biological replicates (Student’s t-test; **P < 0.01).

of H3K27me3 methylation in SlJMJ4-OE43 relative to the WT under dark conditions. These results suggest that SlJMJ4 promotes dark-induced leaf senescence, dependent on H3K27me3 demethylation of senescence regulation-related genes.

SlJMJ4 promotes ABA-induced leaf senescence in tomato

It is well known that ABA is involved in the onset and progression of leaf senescence [28]. Our results showed that dark incubation resulted in higher accumulation of ABA in SlJMJ4-OE43 and SlJMJ4-OE50 lines than in WT lines (Fig. 3g). We speculated that SlJMJ4-promoted leaf senescence is related to ABA, and we therefore investigated the role of SlJMJ4 in regulating tomato leaf senescence in response to ABA. Leaves from SlJMJ4-OE43 and SlJMJ4-OE50 lines showed a more severe senescence phenotype than the WT and had clearly faded from green to yellow at 8 d after ABA treatment (Fig. 6a). Consistent with their phenotypes, the leaves of WT plants retained higher chlorophyll contents (Fig. 6b) and Fv/Fm values (Fig. 6c) than those of SlJMJ4-OE plants.
Figure 4. SlJMJ4 affects dark-induced leaf senescence. a Phenotypes of detached young leaves from 2-month-old WT, SlJMJ4-OE43, and SlJMJ4-OE5 plants at 12 d after dark incubation at 24°C. For the control, leaves were incubated under continuous white light at 24°C. b Chlorophyll contents of WT, SlJMJ4-OE43, and SlJMJ4-OE5 leaves at 12 d after dark incubation or under continuous white light (control). Chlorophyll contents were measured with a SPAD meter. c Ion leakage from detached leaves of WT, SlJMJ4-OE43, and SlJMJ4-OE5 plants at 12 d after dark incubation or under continuous white light (control). d, e Chlorophyll fluorescence images (d) and Fv/Fm (e) of 2-month-old WT, SlJMJ4-OE43, and SlJMJ4-OE50 leaves during dark incubation. f Expression levels of SAGs and chlorophyll degradation genes in detached leaves from 2-month-old WT and SlJMJ4-OE43 plants at 12 d after dark incubation or under continuous white light (control). ACTIN was used as an internal control for qRT-PCR normalization. In b, c, e, and f, the values are shown as the mean ± SE of three biological replicates (Student’s t-test; **P < 0.01).

qRT-PCR analysis showed that the expression levels of SAGs (SISAG12 and SISAG113), chlorophyll degradation-related genes (SISGR1, SINYC1, SIPHO, and SIPAO), and transcription factor genes (SINAP2, SIORE1, and SIENU4) were significantly upregulated in the SlJMJ4-OE43 line compared with the WT (Fig. 6d). Interestingly, the expression levels of many genes related to ABA synthesis (NCED1 and NCED3) and signaling (SlPYP14, SlPYP6, and
Figure 5. SlJMJ4 activates the transcription of SISAG12, SISAG113, SINAP2, and SIORE1 by reducing their H3K27me3 levels. a–d Genome browser visualization of the binding sites of SISAG113 (a), SISAG12 (b), SINAP2 (c), and SIORE1 (d) genes detected by ChIP-seq in the SlJMJ4-OE line. Black box, exon; Black line, intron; Grey box, UTR. Black bar, 200 bp. P1, P2, and P3 indicate different primer pairs. e–h ChIP-qPCR analysis of H3K27me3 methylation status at the SISAG113 (e), SISAG12 (f), SINAP2 (g), and SIORE1 (h) loci in detached leaves from WT and SlJMJ4-OE plants at 12 d after dark incubation or under continuous white light (control). ACTIN was used as an internal reference for ChIP-qPCR. Three biological replicates were used. The data are presented as the mean ± SE of three replicates (Student’s t-test, **P < 0.01).
Figure 6. SlJMJ4 affects ABA-induced leaf senescence. **a** Phenotypes of detached leaves from 2-month-old SlJMJ4-OE and WT plants at 8 d after ABA treatment. **b** Total chlorophyll content in SlJMJ4-OE and WT leaves at 8 d after ABA treatment. **c** Chlorophyll fluorescence Fv/Fm values of SlJMJ4-OE and WT leaves at 8 d after ABA treatment. **d** Relative expression levels of SAGs, chlorophyll degradation genes, and ABA-related genes at 8 d after ABA treatment. ACTIN was used as the internal control. The data are presented as the mean ± SE of three replicates (Student’s t-test, **P < 0.01).
and signaling, SINCED3 and SIABI5, were also the direct targets of SlJMJ4 (Fig. 7a–b; Supplementary Table S1). Three different loci (P1, P2, and P3) were selected with the Integrative Genomics Viewer (IGV) to explore the binding of SlJMJ4 to the loci of SISAG12, SISAG113, SINAP2, SIORE1 (Fig. 5a–d), SINCED3, and SIABI5 (Fig. 7a–b) by ChIP-qPCR. The results confirmed that SlJMJ4 bound directly to the loci of these genes. Moreover, the binding of SlJMJ4 at the loci of SISAG12, SISAG113, SINAP2, SIORE1, SINCED3, and SIABI5 were intensiﬁed in SlJMJ4-OE43 leaves after ABA treatment, indicating that ABA treatment resulted in increased binding of SlJMJ4 at target gene loci (Fig. 7c–h).

In addition, we also analyzed H3K27me3 methylation status at the SISAG12, SISAG113, SINAP2, SIORE1, SINCED3, and ABI5 loci. The H3K27me3 levels of these genes were signiﬁcantly lower in SlJMJ4-OE43 leaves than in WT leaves at 8 d after ABA treatment (Fig. 7i–n). Taken together, these results indicated that SlJMJ4 increased sensitivity to ABA by binding to many key genes related to ABA synthesis and signaling, transcription regulation, and senescence, thereby promoting their H3K27me3 demethylation. More importantly, our research established a close relationship between SlJMJ4-mediated H3K27me3 demethylation and the ABA response for regulating leaf senescence in tomato.

Discussion

Histone demethylases play important roles in regulating histone methylation level and gene expression in plants [15]. In the model plants Arabidopsis and rice, the involvement of histone demethylase in regulating physiological processes has been extensively elucidated. However, relatively few histone demethylases have been characterized in other plant species. Here, we characterized the expression, subcellular localization, and histone demethylation activity and speciﬁcity of SlJMJ4 and further elucidated its possible role in the regulation of tomato leaf senescence.

SlJMJ4 is an H3K27 demethylase

Histone lysine methylation is an important epigenetic mark that is crucial for regulating diverse biological processes [29]. Histone lysine methylation mainly occurs at K4, K9, K27, and K36 in histone H3, and it is dynamically regulated by histone demethylases and methyltransferases [15]. The involvement of H3K9 and H3K4 demethylases in biological processes has been widely reported in Arabidopsis and rice [15], whereas relatively few H3K27 demethylases have been characterized. Arabidopsis REF6 (JM12) was the ﬁrst H3K27me2/3 demethylase reported in plants [30]. Subsequently, ELF6 (JM11), JM13, and JM30 were also conﬁrmed as H3K27 demethylases [31–33]. In the present study, we identiﬁed SlJMJ4, which was classiﬁed into the KDM4/JHD3 subgroup, as an H3K27 demethylase from tomato. Enzymatic activity analyses in vitro demonstrated that SlJMJ4 speciﬁcally demethylates histone H3K27me2/3 (Fig. 2b). Moreover, overexpression of SlJMJ4 in tomato plants reduced the global levels of H3K27me2/3 (Fig. 2a). Therefore, SlJMJ4 acts as an H3K27me2/3 demethylase in tomato.

SlJMJ4 promotes the transcription of functional and regulatory genes related to senescence by reducing their H3K27me3 levels during dark-induced leaf senescence

Among the different epigenetic mechanisms, histone methylation modiﬁcations have been extensively investigated and characterized. In most eukaryotic genomes, a large proportion of chromatin is enriched with H3K27me3 [33]. There is mounting evidence that histone lysine methylation modiﬁcations regulated by H3K27 demethylases are involved in plant developmental and physiological processes, especially the ﬂoral transition. The H3K27me3 demethylases REF6 [34] and ELF6 [16, 35] directly regulate the central ﬂowering regulators FLOWERING LOCUS T (FT) and FLOWERING LOCUS C (FLC) and control the transition from vegetative growth to ﬂowering in Arabidopsis. The elf6 and ref6 mutants display early and late ﬂowering phenotypes, respectively. Similarly, the mediation of H3K27 demethylation at the FLC locus by JM30 and its homolog JM32 contributes to the thermal stability of ﬂowering at elevated temperatures [32]. In addition, the H3K27me3 demethylase JM705 is recruited by WUSCHEL-related homeobox 11 to promote gene expression during shoot growth in rice [36].

Currently, there is little information about the role of H3K27 demethylase in regulating plant senescence. The H3K27me3 demethylase REF6 is suggested to promote leaf senescence by activating numerous functional and regulatory genes related to senescence in Arabidopsis [21]. In this study, we generated SlJMJ4-overexpressing transgenic tomato plants and found that they had an obvious premature senescence phenotype, with earlier de-greening, lower chlorophyll content, and higher yellowing ratio than the WT at 12 weeks (Fig. 3). Moreover, when subjected to dark conditions for 12 d, SlJMJ4-overexpressing leaves showed lower chlorophyll content, lower chlorophyll ﬂuorescence, and higher ion leakage compared with the WT. These results indicate that SlJMJ4 promotes leaf senescence in an age-dependent manner and under dark conditions. SlJMJ4 had high homology with AtJM13 and OsJM706 (Fig. S1b). AtJM13 is an H3K27me3 demethylase and a ﬂowering inhibitor [24], whereas OsJM706 has H3K9me2/3 demethylase activity and regulates rice ﬂowering [25]. Neither of these histone demethylases has been reported to regulate leaf senescence. We have constructed SlJMJ4, SlJMJ6 [18], and SlJMJ7 (data not shown) overexpression lines, and only the SlJMJ4 overexpression line exhibited the premature leaf senescence phenotype. SlJM6 delays tomato fruit ripening, and SlJMJ7 promotes tomato fruit ripening, but neither promote the premature leaf senescence phenotype. It appears that different SlJMJs may have speciﬁc biological functions.
Figure 7. ChIP-qPCR analysis of the binding and H3K27me3 methylation status of SlJMJ4 at its target gene in response to ABA. 

(a) Genome browser visualization of the binding sites of SlABI5 (a) and SlNCED3 (b) genes detected by ChIP-seq in the SlJMJ4-OE line. Black box, exon; Black line, intron; Grey box, UTR. Black bar, 200 bp. P1, P2, and P3 indicate different primer pairs. 

(c–h) ChIP-qPCR analysis of the binding of SlJMJ4 at the loci of SlSAG113 (c), SlSAG12 (d), SlNAP2 (e), SlORE1 (f), SlSINCE3 (g), and SlABI5 (h) in detached leaves from 2-month-old SlJMJ4-OE and WT plants at 8 d after ABA treatment. An anti-GFP antibody was used for immunoprecipitation, and IgG was used as the negative control.

(i–n) ChIP-qPCR analysis of H3K27me3 methylation status at the loci of SlSAG113 (i), SlSAG12 (j), SlNAP2 (k), SlORE1 (l), SlABI5 (m), and SlNCED3 (n) in detached leaves from 2-month-old SlJMJ4-OE and WT plants at 8 d after ABA treatment. ACTIN was used as an internal reference for ChIP-qPCR. Three biological replicates were used. The data are presented as the mean ± SE of three replicates (Student’s t-test, **P < 0.01).
Senescence begins with chlorophyll degradation, involving a number of chlorophyll degradation–related genes, SAGs, and regulators [10]. A set of key genes, including pheophytin pheophorbide hydrolase (PPH), STAY-GREEN 1 (SGR1), NON-YELLOWING 1 (NYE1), chlorophyll catabolite reductase (RCCR), and pheophorbide a oxygenase (PAO), are involved in this process [10]. In addition, as revealed by genomic, genetic, metabolomic, proteomic, and transcriptomic research, leaf senescence is dynamically regulated by numerous SAGs [37]. Furthermore, several transcription factors, including OsNAP [38], ORE1 [3], AtNAP2 [2, 39], and
SlJMJ4 affects ABA-induced leaf senescence by reducing H3K27me3 levels of ABA-related genes to regulate their transcription

Senescence is triggered by various endogenous and environmental signals. The plant hormone ABA plays an important role in the onset and progression of leaf senescence [28]. Under external stimuli, ABA is rapidly synthesized and then sensed by ABA receptors, ultimately activating a cascade of transcription factors [40]. Previous work suggests that ABA stimulates a set of SAGs by transcriptional regulation in ABA signaling pathways to drive leaf senescence [44]. However, the mechanism by which ABA induces SAG expression at the onset of leaf senescence still remains to be elucidated.

Recent studies have revealed that epigenetic modification is implicated in leaf senescence in response to ABA. ABA induces leaf senescence by decreasing H3K27me3 of SAGs [45]. ABREs (ABA-responsive elements) are subjected to H3K27me3 modification via polycomb repressive complex 2 (PRC2) [46], as well as the H3K27 trimethyltransferase CLF and SWN [47]. The clf swn double mutants are hypersensitive to ABA, with decreased H3K27me3 levels at SAG gene loci compared with WT plants [48]. More recently, Wang et al. [49] reported that the H3K4 demethylase JM17 is recruited to ABI5 chromatin by interacting with WRKY40 upon ABA exposure. In this study, SlJMJ4 increased the sensitivity of tomato leaves to ABA, with accelerated leaf senescence in SlJMJ4-OE leaves compared with WT leaves [48]. Upon ABA treatment, the H3K27me3 level at the SNCED3 locus was greatly decreased in SlJMJ4-OE43 plants compared with the WT (Fig. 7), consistent with upregulated SNCED3 expression. ABI5 is a downstream transcription factor in the ABA signaling pathway and plays a key positive regulatory role in the ABA response [49]. In Arabidopsis, ABI5 binds to the promoter of ORE1 and promotes its expression and leaf senescence [50]. Overexpression of ORE1 triggers early senescence by controlling downstream SAGs, whereas its inhibition delays senescence in Arabidopsis [51]. In tomato, SlABI5 regulates SISGRL expression by directly binding to the ABRE cis-element to promote chlorophyll degradation [44]. In addition, SIRNA2 binds directly to the promoters of SISAG13, SISGRL, SIPAO, and other downstream targets to activate their expression and promote leaf senescence [52]. In the present study, in response to ABA, SlJMJ4 upregulated the expression of SIABI5, SlSAG12, SlSAG13, and SlSISGRL by directly recruiting chromatin via WRKY40, thereby activating the ABA signaling pathway.

Based on the above results, we proposed a model to explain the involvement of SlJMJ4 in regulating tomato leaf senescence in response to ABA (Fig. 8). After ABA treatment, H3K27me3 levels of SNCED3 genes decreased in SlJMJ4-OE43 lines, thus promoting ABA synthesis. The H3K27 demethylation of SIABI5 by SlJMJ4 is induced by ABA, which promotes ABA signal transduction and activates the expression of SIABI5 and downstream SISGRL and SISAG13, thereby stimulating leaf senescence. Moreover, SlJMJ4 directly upregulates the expression of SlSAG12, SlSAG13, and SlSISGRL genes via H3K27me3 demethylation. In summary, SlJMJ4 is involved in the ABA-induced senescence of tomato leaves by binding to many genes related to ABA synthesis and signal transduction, promoting their H3K4me3 demethylation.

Histone demethylases act by regulating gene transcription and chromatin structure. However, it is not clear how the histone demethylases recognize and bind to specific genomic sites. Cui et al. [30] and Li et al. [14]...
Figure 8. A proposed model to explain the involvement of SlJMJ4 in regulating tomato leaf senescence in response to ABA. After ABA treatment, H3K27me3 levels of SlNCED3 genes decreased in SlJMJ4-OE43 lines, thus promoting ABA synthesis. The H3K27 demethylation of SlABI5 by SlJMJ4 is induced by ABA, which promotes ABA signal transduction and activates the expression of SlABI5 and downstream SlORE1 and SAG genes, thereby stimulating leaf senescence. Moreover, SlJMJ4 directly upregulates the expression of SlORE1, SlNP2, SlSAG113, and SlSAG12 genes via H3K27me3 demethylation. In summary, SlJMJ4 is involved in the ABA-induced senescence of tomato leaves by activating the transcription of many genes associated with ABA synthesis and signal transduction via removal of H3K27me3. The solid arrows indicate confirmed processes, whereas the dotted arrows indicate speculation based on our work and previous studies.

Materials and methods

Plant material and growth conditions
Tomato (Solanum lycopersicum L. cv. Ailsa Craig) was used as the wild type (WT), and transgenic lines were generated in the WT background. The WT and transgenic lines were grown under long-day conditions (16 h light/8 h dark) with 65–70% relative humidity at 24°C.

Sequence analysis
Sequence analyses of SlJMJ4s used the Conserved Domain Database (CDD) [54] (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the Simple Modular Architecture Tool (SMART) (http://smart.embl-heidelberg.de) [55]. Protein alignment was performed using phmmer at Ensembl Plants (http://plants.ensembl.org/hmmer/index.html) [56] and Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo) [57]. The Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/color_aligncons.html) [58] was used to highlight conserved regions within the alignments.

Subcellular localization
An SlJMJ4-pSAT6-GFP fusion vector was constructed, and the fusion vector or a control vector was co-transformed with an mCherry vector into Arabidopsis protoplasts as previously described by Yoo et al. [59]. After 2 d of incubation, GFP and mCherry fluorescence were observed under a fluorescence microscope (Leica SP8 STED 3X) and detected at 488 and 590 nm, respectively.

Vector construction and transgenic plant generation
The full-length cDNA of SlJMJ7 was subcloned into the pBI121-GFP vector using the In-Fusion HD Cloning Kit (TaKaRa, Japan). Transgenic plant generation of SlJMJ4 overexpression lines was carried out as previously described by Li et al. [18]. In brief, the constructs were transformed into Agrobacterium tumefaciens GV3101, which was subsequently used to infiltrate young cotyledon sections at 23°C under a 16-h light/8-h dark photoperiod. Transformants were selected based on their resistance to kanamycin. T2 homozygous progeny were used for phenotypic and molecular characterization.

Quantitative RT-PCR analysis (qRT-PCR)
The RNeasy Plant Mini Kit (Qiagen) was used to extract total tissue RNA in accordance with the manufacturer’s instructions. qRT-PCR was performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems) using SYBR Green (Applied Biosystems). ACTIN (Solyc03g078400) was used as the reference gene for data normalization. Gene-specific primers for qRT-PCR are listed in Supplementary Table S3.
Histone H3K27 demethylation assay in vitro and in vivo

For in vitro demethylation assays, SJMJ4-GST fusion proteins were purified using glutathione sepharose 4B (GE Healthcare). Afterwards, histone demethylase activity was analyzed as previously described [18]. In brief, the purified GST-tagged SJMJ14 (4.0 μg) was incubated with calf thymus histones (Sigma) in a reaction buffer containing 150 mM NaCl, 80 μM Fe(NH4)2(SO4)2, 50 mM Tris–HCl (pH 7.0), 1 mM α-KG, and 2 mM ascorbic acid for 6 h at 37°C. The reaction was terminated with 10 μM EDTA and subjected to western blotting analysis. For in vitro demethylation assays, histones were extracted from 2-month-old leaves of SJMJ4-OE and WT plants with the EpiQuik Total Histone Extraction Kit (Epigentek, Farmingdale, NY, USA) and analyzed by western blotting. The antibodies used in this experiment were from Abcam: H3K4me1 (ab176877, 1:3000 dilution), H3K4me2 (ab11946, 1:3000 dilution), H3K4me3 (ab8580, 1:3000 dilution), H3K9me1 (ab9045, 1:3000 dilution), H3K9me2 (ab1220, 1:1000 dilution), H3K9me3 (ab8898, 1:1000 dilution), H3K27me1 (ab115068, 1:3000 dilution), H3K27me2 (ab24684, 1:3000 dilution), H3K27me3 (ab6002, 1:3000 dilution), H3K36me1 (ab176920, 1:3000 dilution), H3K36me2 (ab176921, 1:3000 dilution), H3K36me3 (ab9050, 1:3000 dilution), and H3 (ab1791, 1:5000). H3 was used as a loading control.

Chromatin immunoprecipitation (ChIP) and ChIP-seq analysis

ChIP was performed as previously described with slight modifications [60]. Leaves from 2-month-old tomato plants were cross-linked with 1% formaldehyde. The extracted chromatin was sheared to lengths of 100–300 bp by sonication, then immunoprecipitated using anti-GFP antibody (Abcam, ab290, 1:200 dilution) and anti-GFP (Abcam, ab290, 1:200 dilution) antibodies, IgG (Millipore, 12-370, 1:500 dilution), and high performance liquid chromatography (HPLC, Agilent 1290). Ion leakage was determined as described previously [63].

Measurement of senescence parameters

The total chlorophyll content was measured with a SPAD-502 chlorophyll meter (Konica-Minolta). Chlorophyll fluorescence was determined using a modulated fluorometer (OS-500, Opti-Sciences) and expressed as Fv/Fm [61]. ABA was obtained by the method of Forcat et al. [62] and measured using tandem mass spectrometry (MS/MS, Applied Biosystems 6500 Quadrupole Trap) and high performance liquid chromatography (HPLC, Agilent 1290). Ion leakage was determined as described previously [63].

ChIP-qPCR

Tomato leaves subjected to dark treatment or ABA treatment were used for ChIP as mentioned above. For ChIP-qPCR, 95% of the chromatin was used for immunoprecipitation with anti-H3K27me3 (Millipore 07-473, 1:200 dilution), IgG (Millipore, 12-370, 1:500 dilution), and anti-GFP (Abcam, ab290, 1:200 dilution) antibodies, and the remaining 5% was used as the input control. The input and precipitated DNA were subjected to qRT-PCR. Gene-specific primers for qRT-PCR are listed in Supplementary Table S3.

Statistical analysis

Data are expressed as the mean ± standard error (SE). Differences among treatments were determined by ANOVA followed by Student’s t-test.

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Author contributions

Xiaochun Ding: conceptualization, data curation, formal analysis, experiments, methodology, software, and writing - original draft. Dandan Zhang, Dachuan Gu, Zhiwei Li, Hanzhi Liang, and Hong Zhu: experiments and methodology. Yueming Jiang: review of the manuscript. Xuewu Duan: funding acquisition, project administration, supervision, and writing - review and editing. All authors read and approved the final manuscript.

Data availability

Both processed and raw ChIP-seq data are stored at the NCBI GEO repository (http://www.ncbi.nlm.nih.gov/geo; accession number GSE177487). The antibodies used in this article are commercial antibodies, and antibody information (e.g., manufacturer, including sufficient address details to enable contact) are provided in the Materials and Methods. The supplementary data that
support the findings of this study are openly available in the figshare public repository at https://figshare.com/s/e0c8c9a7667870413e221.

Conflict of interest
The authors declare no competing interests.

Supplementary data
Supplementary data are available at Horticulture Research Journal online.

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