SIWRKY45 interacts with jasmonate-ZIM domain proteins to negatively regulate defense against the root-knot nematode Meloidogyne incognita in tomato

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Abstract
Parasitic root-knot nematodes (RKNs) cause a severe reduction in crop yield and seriously threaten agricultural production. The phytohormones jasmonates (JAs) are important signals regulating resistance to multiple biotic and abiotic stresses. However, the molecular mechanism for JAs-regulated defense against RKNs in tomato remains largely unclear. In this study, we found that the transcription factor SIWRKY45 interacted with most JA-ZIM domain family proteins (JAZs), key repressors of the JA signaling. After infection by the RKN Meloidogyne incognita, the slwrky45 mutants exhibited lower gall numbers and egg numbers per gram of roots than wild type, whereas overexpression of SIWRKY45 attenuated resistance to Meloidogyne incognita. Under M. incognita infection, the contents of jasmonic acid (JA) and JA-isoleucine (JA-Ile) in roots were repressed by SIWRKY45-overexpression. Furthermore, SIWRKY45 bound to and inhibited the promoter of the JA biosynthesis gene ALLENE OXIDE CYCLASE (AOC), and repressed its expression. Overall, our findings revealed that the SIJAZ-interaction protein SIWRKY45 attenuated RKN-regulated JA biosynthesis and repressed defense against the RKN M. incognita in tomato.

Introduction
The phytoparasitic root-knot nematodes (RKNs Meloidogyne spp.) are extensively distributed throughout the world. Among them, Meloidogyne incognita is known as one of the most harmful RKNs [1]. Infective juveniles (J2s) of RKNs infect roots of plants, parasitize vascular cylinders, and stimulate roots to develop giant cells and form root-knots (galls) [2–4]. These galls destroy the normal physiological activities of the roots, hinder transport of water and nutrients, reduce host growth and yield, and even lead to host plant death [5, 6].

Jasmonates (JAs), a class of phytohormones, comprise jasmonic acid (JA) and its derivatives including jasmonic acid-isoleucine (JA-Ile), and methyl-jasmonic acid (MeJA) [7]. The study of JA signal transduction began with the screening of Arabidopsis coronatine (a JA-Ile mimic)-insensitive mutants and cloning of COI1 (CORONATINE INSENSITIVE 1) [8]. COI1 forms the SCFCOI1 E3 ligase along with ARABIDOPSIS SKP-LIKE1 (ASK1)/ASK2, Cullin and RBX1 [8, 9]. JA-ZIM domain proteins (JAZs) are repressors of the JA signaling pathway [10–12], and they interact with and repress diverse downstream factors [13, 14]. COI1 serves as the primary JA receptor [15], and interacts with JAZs to form the COI1-JAZ coreceptor complex that effectively perceives bioactive JA forms (e.g. JA-Ile) [16–18]. JA signals trigger degradation of JAZ repressors via the SCFCOI1-26S proteasome pathway, and activate diverse JA responses [10, 11, 19].

Previous studies demonstrated that exogenous application of JA enhances resistance to RKNs in tomato [20]. Consistently, spr2, a tomato JA-deficient mutant, displayed a RKN-susceptible phenotype compared with wild type [21, 22]. Recent studies revealed that some factors affect JA-regulated defense against the RKN M. incognita in tomato. For instance, miR319 and the transcription factor TCP4 regulate resistance to M. incognita by affecting JA contents [23]. The bHLH-type JA signaling transcription factor SIMYC2 participates in crosstalk between JA, strigolactone (SL), and abscisic acid (ABA) to inhibit resistance to M. incognita [24]. SICSN4 and SICSN5 interact with SIJAZ2 to positively regulate defense against M. incognita [25]. Although JAs control tomato defense against RKNs, the underlying molecular mechanism has not been fully explored, and remains to be elucidated.

WRKY transcription factors modulate plant defense against abiotic and biotic stresses [26–28]. The WRKY family in tomato contains 83 members [29]. SIWRKY70 confers resistance to aphids and the RKN Meloidogyne javanica, and its transcript level is inducible by salicylic acid (SA) but suppressed by JA [30]. Overexpression of SIWRKY3 enhances resistance to the RKN M. javanica, whereas loss of function of SIWRKY3 causes susceptibility [31]. SIWRKY45 represses tomato resistance to M. javanica, regarding the larger numbers of galls and females in roots with Agrobacterium rhizogenes-mediated SIWRKY45-overexpression [32].
Although SlWRKYs control tomato defense against RKNs, the regulatory mechanism is still unclear.

Here, we provided deep insights into the molecular mechanism by which SlWRKY45 negatively regulated defense against the RKN M. incognita. SlWRKY45 physically interacted with most SlJAZ members (SlJAZ1, SlJAZ2, SlJAZ3, SlJAZ4, SlJAZ5, SlJAZ6, SlJAZ7, and SlJAZ11). Loss of function of SlWRKY45 enhanced resistance to the RKN M. incognita, whereas overexpression of SlWRKY45 decreased defense against M. incognita. Furthermore, SlWRKY45 overexpression reduced the contents of JA and JA-Ile under M. incognita infection, whereas SlWRKY45 bound to the promoter of the JA biosynthesis gene ALLENE OXIDE CYCLASE (AOC) and repressed its expression. Our results provide evidence that SlWRKY45 participates in both JA signaling and biosynthesis pathways, and inhibits resistance to M. incognita in tomato.

**Results**

**SlJAZs interact with SlWRKY45**

To explore the molecular basis of JA pathway in regulation of tomato defense against M. incognita, we sought to identify potential downstream transcription factors of SlJAZ repressors using the yeast two-hybrid (Y2H) system. We first analysed expression levels of SlJAZs at 1, 3, 7, and 14 d after M. incognita infection, and found that the SlJAZ7 expression was notably increased at these time points (Fig. S1, see online supplementary material). SlJAZ7 was selected as a bait and ligated with DNA binding domain (BD) in pLexA to screen a cDNA library of RKN-infected tomato. The WRKY transcription factor SlWRKY45 was identified as a prey of SlJAZ7-1 interaction (Fig. S1, see online supplementary material). The editing rate was 69.2% (Fig. S5B, see online supplementary material). Three independent T0 transgenic lines (slwrky45-cr-1 to slwrky45-cr-13) were obtained through Agrobacterium-mediated transformation (Fig. S5A, see online supplementary material). Sequencing-based genotyping showed that the slwrky45 mutations were homozygous in slwrky45-cr-1 and slwrky45-cr-2, heterozygous in slwrky45-cr-10 and slwrky45-cr-13, bi-allelic in slwrky45-cr-3, slwrky45-cr-9 and slwrky45-cr-11, and chimeric in slwrky45-cr-5 and slwrky45-cr-7, and it was wild type (WT) in the remaining four lines (Fig. S5A, see online supplementary material). The editing rate was 69.2% (Fig. S5B, see online supplementary material).

The two representative T0 homozygotes, slwrky45-cr-1 (16-bp and 1-bp deletions, respectively, in the first and second target) and slwrky45-cr-2 (3-bp and 1-bp deletions, respectively, in the first and second target) were chosen for further study (Fig. S5A, see online supplementary material). We first analysed the presence and absence of the slwrky45 mutations and Cas9 in 15 T1 generation plants of slwrky45-cr-1 and slwrky45-cr-2, respectively. As shown in Fig. S6 (see online supplementary material), all the detected T1 generation contained the same slwrky45 mutations as their corresponding parental T0 lines, suggesting that these mutations were stably inherited by their T1 progeny. Meanwhile, Cas9-free T1 homozygotes were identified (three plants from slwrky45-cr-1 and four plants from slwrky45-cr-2). Furthermore, we investigated the three most probable off-target sites of the two single guide RNAs in the slwrky45-cr-1 and slwrky45-cr-2 T1 plants, and found that no mutations occurred at these off-target sites (Fig. S7 and Table S1, see online supplementary material). Therefore, Cas9-free T2
Figure 1. SIJAZ proteins interact with SIWRKY45. **A-B** Yeast two-hybrid (Y2H) assays to assess interactions of SIJAZs with SIWRKY45. SIJAZs and SIWRKY45 were fused to BD domain in pLexA (marked as BD-SIJAZs or BD-SIWRKY45) or B42AD domain in pB42AD (marked as AD-SIJAZs or AD-SIWRKY45). **C** Interactions of SIJAZs and SIWRKY45 were detected by firefly luciferase (LUC) complementation imaging (LCI) assays. SIJAZ1, SIJAZ2, SIJAZ3, SIJAZ4, SIJAZ7, SIJAZ11, and SIWRKY45 were fused with nLUC or cLUC (N or C-terminal fragments of LUC) to produce SIJAZ1-nLUC, SIJAZ2-nLUC, SIJAZ3-nLUC, SIJAZ4-nLUC, SIJAZ7-nLUC, SIJAZ11-nLUC, and cLUC-SIWRKY45, respectively. Luciferase activities were evaluated at 50 h after the infiltration of corresponding *Agrobacterium* strains in leaves of *N. benthamiana*. **D** Pull-down assays show that SIJAZ7 and SIJAZ11 associate with SIWRKY45. MBP and MBP-SIWRKY45 were immobilized on amylose resin, and incubated with transiently expressed myc-SIJAZ7 and myc-SIJAZ11 proteins. The samples were detected with an anti-myc antibody. **E** Bimolecular fluorescence complementation (BiFC) analyses show interaction of SIJAZ7/11 and SIWRKY45. SIJAZ7, SIJAZ11, and SIWRKY45 were fused with nYFP or cYFP (N or C-terminal parts of YFP), respectively. YFP signals were observed at 50 h after the expression of corresponding combinations of *Agrobacterium* strains in leaves of *N. benthamiana*. 

Homozygotes of slwrky45-cr-1 and slwrky45-cr-2 (Fig. S8, see online supplementary material) were used for ensuing studies. The CM wild type, slwrky45-cr-1, and slwrky45-cr-2 plants were inoculated with *M. incognita*. At 7 d and 35 d after inoculation, the number of galls for each root were counted. The results in Fig. S9A and B (see online supplementary material) showed that, at 7 d post inoculation, the gall numbers per gram of roots were significantly lower in slwrky45-cr-1 and slwrky45-cr-2
Figure 2. The N-terminal parts of SlJAZs mediate interaction between SlJAZs and SIWRKY45. A Diagrams of SlJAZ7 and SlJAZ11 domain constructs. Red and yellow boxes represent the ZIM and Jas domains, respectively. B Y2H assays showing interactions of SlJAZ7 and SlJAZ11 domain constructs with SIWRKY45. The corresponding domains of SlJAZ7 and SlJAZ11, and SIWRKY45 were fused with AD or BD domains, respectively. C-D Interactions of the N-terminal parts of SlJAZ7 (C) and SlJAZ11 (D) with SIWRKY45 were analysed by LCI assays. N-terminal fragments of SlJAZ7 and SlJAZ11, and SIWRKY45 were in fusion with nLUC and cLUC, respectively. Luciferase activities were determined at 50 h after the injection of corresponding Agrobacterium strains in leaves of N. benthamiana.

compared with those in WT. Furthermore, we found that at 35 d post inoculation, the gall numbers per plant, and gall numbers and egg numbers per gram of roots in slwrky45-cr mutants were also significantly lower compared with those in WT (Fig. 4A–C), consistently indicating that SIWRKY45 plays a negative role in defending against M. incognita. PLANT DEFENSE FACTOR (PDF) and PROTEINASE INHIBITOR 2 (PI-2) are two defensive genes against pathogens, including M. incognita [24]. SI PDF and SIPI-2 expression was responsive to M. incognita infection as shown in Fig. 4D and E. Consistent with the increased resistance of slwrky45-cr plants, the expression levels of SI PDF and SIPI-2 were obviously higher in slwrky45-cr roots with the RKN M. incognita infection compared with those in the M. incognita-infected WT roots (Fig. 4D and E).

Overexpression of SIWRKY45 reduces resistance to M. incognita in tomato

We further generated SIWRKY45-overexpressing lines of tomato. Flag-SIWRKY45-OE-5 and flag-SIWRKY45-OE-13 with approximately 39-fold and 28-fold of the WT level regarding SIWRKY45 expression, respectively, were used as representatives (Fig. S9C, see online supplementary material). The CM wild type and flag-SIWRKY45-OE plants were inoculated with M. incognita. The gall numbers per plant in flag-SIWRKY45-OE roots at 7 d after infection, and the gall numbers per plant, gall numbers and egg numbers per gram of roots in flag-SIWRKY45-OE plants at 35 d after infection were larger than those in the infected WT roots at 7 d, and 35 d, respectively (Figs S9D (see online supplementary material) and SA–C). We further analysed the transcript levels of SI PDF and SIPI-2 in the roots of WT and flag-SIWRKY45-OE lines with M. incognita infection. As shown in Fig. 5D and E, the expression of SI PDF and SIPI-2 in the roots of flag-SIWRKY45-OE lines at 1 d, 3 d, 7 d, and 14 d after infection was significantly lower than those in the infected WT roots.

Altogether, the results in Figs. 4, 5, and S9 (see online supplementary material) demonstrate that SIWRKY45 acts as a repressor to regulate resistance to M. incognita in tomato.

SIWRKY45 overexpression attenuates RKN-affected JAs biosynthesis in tomato

To further explore the mechanism of SIWRKY45 in regulating the resistance to M. incognita, we examined the concentrations of JA and JA-Ile in the roots of WT and SIWRKY45-overexpressing plants (flag-SIWRKY45-OE-5 as a representative) without or with M. incognita inoculation. In contrast to those in WT roots without M. incognita infection, the contents of JA and JA-Ile in a 1 d, 3 d, 7 d, and 14 d after infection was significantly lower than those in the infected WT roots. In addition, the CM wild type and flag-SIWRKY45-OE-5 plants were treated with MeJA, and 24 h later, these plants were infected with M. incognita, and 24 h later, these plants were infected with M. incognita for 7 d and 35 d. The results in Figs. S10 (see online supplementary material) and 6C–E showed that MeJA
Figure 3. SIWRKY45 is localized to the nucleus and is inducible by the RKN Meloidogyne incognita infection. A, SIWRKY45 is localized to the nucleus. Agrobacterium strains GV3101 carrying the empty vector or GFP-fused SIWRKY45 vector were injected into leaves of N. benthamiana to transiently express GFP and GFP-fused SIWRKY45 protein. GFP signals were detected using a confocal microscope at 50 h after injection. B, Relative expression level of SIWRKY45 in the roots of CM wild type (WT) at the indicated time points after infection without (control) or with Meloidogyne incognita. Values represent means (±SD) of three independent biological replicates. Significant differences between Meloidogyne incognita-infected roots of CM and the corresponding control were analysed by Student’s t test (**P < 0.01) and indicated with asterisks.

SlWRKY45 binds to and represses the JA biosynthesis gene SlAOx

A previous study showed that two JA biosynthesis genes LIPIDXYGENASE D (LOXD) and ALLENE OXIDE CYCLASE (AOx) were induced by the RKN M. incognita infection in tomato [25]. Thus, we explored whether the decreased JAs contents in M. incognita-infected SIWRKY45-overexpressing plants were due to reduced expression of SlLOXD or SlAOx under M. incognita infection. To demonstrate this, we analysed the transcript levels of SlLOXD and SlAOx, and discovered that, in response to M. incognita infection, the expression levels of SlAOx were lower in SIWRKY45-overexpression lines compared with those in WT (Fig. 7A), while SlLOXD expression was not affected (Fig. S11, see online supplementary material).

We next investigated whether SIWRKY45 was able to bind to and regulate SlAOx. We carried out chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) analysis using M. incognita-infected flag-SIWRKY45-OE-5 transgenic plants, and found that SIWRKY45 bound to the first and second typical WRKY factor target sequences (W-box, TTGACT) in the promoter of SlAOx (Fig. 7B and C). We further examined the regulatory function of SIWRKY45 on the SlAOx promoter using Dual-LUC assays in which the SlAOx promoter drove the luciferase (LUC) reporter gene (SlAOxpro-LUC), and SIWRKY45 controlled by the CaMV35S promoter (35S-SIWRKY45) served as an effector (Fig. 7D). As shown in Fig. 7E, coexpression of SlAOxpro-LUC and 35S-SIWRKY45 in N. benthamiana leaves resulted in a lower LUC/REN ratio than the coexpression of SlAOxpro-LUC and the control 35S-GFP, consistently suggesting that SIWRKY45 obviously represses the promoter and expression of SlAOx. Moreover, we found that the repression activity of SIWRKY45 was attenuated by SlJAZ11 (Fig. 7E), suggesting that SlJAZ11 inhibits the function of SIWRKY45.
Figure 4. The slwrky45 mutant exhibits increased resistance to the RKN Meloidogyne incognita. A–C Gall numbers per plant (A), gall numbers per gram of roots (B), and numbers of eggs per gram of roots (C) in the CM wild type and slwrky45 mutants (slwrky45-cr-1 and slwrky45-cr-2) at 35 d after M. incognita infection. Data represent means (±SD) of 20 plants. Significant differences were analysed by ANOVA with Duncan’s multiple range test (P < 0.05) and indicated with letters.

Discussion

Previous studies have indicated that WRKY transcription factors associate with JA pathway to exert their biological functions. For example, AtWRKY57 integrates both auxin and JA signaling by interacting with AtIAA29 and AtJAZs, and mediates leaf senescence [34]. AtWRKY51 associates with AtJAZ8/AtJAV1 to comprise a JAV1-JAZ8-WRKY51 complex for controlling defense against insects [35]. SlWRKY31 cooperates with SlVQ15, and participates in JA signaling and resistance to Botrytis cinerea [36]. Nevertheless, the relationship between JA and WRKYs in tomato remains poorly understood. Here, we reveal that SlWRKY45 is involved in both JA biosynthesis and signaling pathways to attenuate resistance to the RKN M. incognita (Figs 1–7).

WRKYs play regulatory roles via interacting with various proteins. AtWRKY50 interacts with AtTGA2 or AtTGA5 to form a protein complex and synergistically activates the expression of the resistance-related gene PATHOGENESIS-RELATED 1 (PR1) [37]. AtWRKY8 recruits AtVQ10 to enhance its binding to target DNA, and promote resistance to B. cinerea [38]. AtVQ9 associates with and decreases the transcriptional activity of AtWRKY8, and modulates tolerance to salt stress [39]. AtWRKY13 interact with SQUAMOSA PROMOTER BINDING-LIKE 10 to antagonistically regulate their transcriptional functions and age-mediated flowering [40]. AtWRKY38 and AtWRKY62 associate with HISTONE DEACETYLASE 19 to participate in defense responses [41]. Here, we demonstrate that SIJAZs act through their N-terminal regions to physically interact with SIWRKY45 using Y2H, LCI, pull-down, and BiFC assays (Figs. 1 and 2).

WRKYs bind to target genes and their own promoters to activate or repress expression through the combination of the WRKY domain and W-box. For instance, AtWRKY57 binds to the W-box region of the AtJAZ1 and AtJAZ5 promoters and activates their expression to repress JA-mediated defense against B. cinerea [42]. AtWRKY51 binds to the promoter regions of the Arabidopsis JA biosynthesis gene ALLENE OXIDE SYNTHASE (AOS) via the W-box sequence [35]. It also associates with AtJAV1 and AtJAZ8 to repressAtAOS expression, which inhibits JAs biosynthesis and controls resistance to insect attack [35]. PcWRKY1 binds to the W-boxes in its own promoter, as well as the PcWRKY3 and PcPR1–1 promoters [43]. Here, our ChIP-qPCR results showed that SIWRKY45 binds to fragments spanning the W-boxes in the promoter of the JA
biosynthesis gene SIAOC (Fig. 7C). Dual-LUC assays suggested that SIWRKY45 attenuates the expression of SIAOC (Fig. 7E). It would be interesting to further investigate whether SIWRKY45 could bind to its own promoter and regulate its own expression.

One previous study reported that SIWRKY45 expression is enhanced within 5 d after M. javanica infection, and is maintained through feeding-site development and gall formation [32]. The phytohormones cytokinin (CK), auxin, and SA induce SIWRKY45 expression, whereas JA inhibits its expression. In response to the RKN M. javanica infection, the roots with transient SIWRKY45 overexpression displayed higher numbers of developed females, gall formation, and overall feeding site area than WT roots. In this study, we found that SIWRKY45 was localized to the nucleus, and was induced at 1 d, 3 d, 7 d, and 14 d after M. incognita infection (Fig. 3). We generated stable SIWRKY45-overexpressing transgenic tomato and slwrky45 mutants, and demonstrated that SIWRKY45 is a negative regulator of tomato defense against M. incognita (Figs. 4, 5, and S9, see online supplementary material). Moreover, we deeply explored and revealed the mechanism that SIWRKY45 interacts with SlJAZs, directly binds to the SIAOC promoter, and inhibits SIAOC expression and JA biosynthesis to reduce defense against the RKN M. incognita (Figs. 1, 2, 6, and 7).

SIMYC2, a master transcription factor, interacts with 11 SlJAZs [44], and plays positive or negative roles to modulate diverse physiological responses in tomato, including positively regulating fruit chilling tolerance [45], resistance to the necrotrophic pathogen B. cinerea [44, 46], growth and developmental processes such as flower formation, fruit set, and fruit shape [47], and negatively controlling resistance to M. incognita by mediating the interplay of SL, ABA, and JA [24].

In our results, we discovered that SIWRKY45 interacts with most SlJAZ proteins, and negatively controls defense against M. incognita (Figs. 1, 4, 5, and S9, see online supplementary material). It remains to explore whether SIWRKY45 also controls other JA-regulated responses in tomato. As shown by the finding that the transcription factors SIMYC2 and SIWRKY45 both repress resistance to M. incognita in tomato [24] (Figs. 4, 5, and S9, see online supplementary material), it remains to investigate whether SIMYC2 and SIWRKY45 target some mutual downstream genes to synergistically or antagonistically regulate their expression,
and control defense responses. Additionally, it will be interesting to isolate the master factors positively modulating JA-mediated tomato defense against RKNs, which will contribute to further understanding the molecular basis of JA-controlled defense responses.

A summary of our findings is shown in Fig. 8. SlJAZ repressors interact with and repress SlWRKY45 to attenuate its function. JAs induce SlJAZs degradation to release SlWRKY45. The released SlWRKY45 binds to and inhibits SlAOC expression to reduce RKN-regulated JAs biosynthesis, and represses defense against the RKN *M. incognita*.

**Materials and methods**

**Plant materials and growth conditions**

Seeds of CM wild type, SlWRKY45-overexpressing plants and slwrky45 mutants were germinated at 28°C on moistened filter paper for 2–3 days, and then grown in a greenhouse (24°C–26°C/16°C–18°C, 16 h light/8 h dark). Seeds of *N. benthamiana* were sown in soil, and grown in a greenhouse (25°C–28°C/16°C–18°C, 16 h light/8 h dark).

**Generation of SlWRKY45-overexpression plants**

The CDS of SlWRKY45 was amplified with specific primers (Table S2, see online supplementary material), and ligated into a reformatory pCAMBIA1300 vector through the SalI and SpeI sites to generate the SlWRKY45-overexpression plasmid (flag-SlWRKY45-OE), in which SlWRKY45 was fused with three flag tags and was driven by the CaMV35S promoter. Through Agrobacterium (GV3101)-mediated cotyledon explant transformation, this construct was transformed into CM wild type. Hygromycin B was used to select the transgenic lines. T3 homozygous SlWRKY45-overexpressing plants were used for further experiments.

**Generation of SlWRKY45 gene-edited plants**

CRISPR/Cas9 technology was used to generate slwrky45 mutants. We used CRISPR-P to choose two sgRNAs that targeted the first and second exons of SlWRKY45 (Fig. S4A, see online supplementary material). The PCR primers included the two target sites and the BsaI site, which are shown in Table S2, see online supplementary material. The PCR fragment was amplified using the plasmid pSG-SIU6 (Biogle GeneTech, Jiangsu, China) as a template to generate the PCR product containing the 8sa I site,
Figure 7. SIWRKY45 binds and represses the expression of SIAOC. A Relative expression level of SIAOC at 1 d, 3 d, and 7 d post infection without (control) or with Meloidogyne incognita in the indicated plants. Data represent means (±SD) of three independent biological replicates. Significant differences were analysed by ANOVA with Duncan’s multiple range test (P < 0.05) and indicated with letters. B Diagram of SIAOC promoter. Red lines represent the regions detected in ChIP-qPCR assays. ChIP-qPCR assays to detect the binding of flag-SIWRKY45 to SIAOC promoter. Chromatin from RKN-infected CM wild type and the flag-SIWRKY45 transgenic plants was immunoprecipitated without (−) or with (+) anti-flag antibody. A promoter of SIACTIN2 was used as a negative control. Data represent means (±SD) of three independent biological replicates. Significant differences were analysed by ANOVA with Duncan’s multiple range test (P < 0.05) and indicated with different letters. C Diagram displaying the constructs used in the Dual-LUC assays in (E). E Dual-LUC assays showing that SIWRKY45 attenuates SIAOC promoter activity, and that SIJAZ11 inhibits this effect. Error bars represent SD (n = 6). Significant differences were analysed by ANOVA with Duncan’s multiple range test (P < 0.05) and indicated with letters.

two targets, gRNA scaffold, and the tomato U6 promoter (Fig. S4B, see online supplementary material). The product was purified and inserted into the pCBSG012 vector (Biogle GeneTech, Jiangsu, China) through the Bsa I site (Fig. S4C–D, see online supplementary material). Through Agrobacterium (GV3101)-mediated cotyledon transformation, this construct was transformed into CM wild type. Hygromycin B was used to select the transformants. slwrky45 mutants of Cas9-free T2 homozygotes were analysed by PCR and sequencing, and used for further experiments.

Analysis of mutation types and off-target mutations

Tomato DNA was extracted using a DNA extraction kit (GeneBette, Beijing, China), and used as a template to amplify the target sites using PrimeSTAR Max DNA polymerase (TaKaRa, Ohtsu, Japan). To analyse mutation types of each T0 line, we cloned the PCR fragments into the pMD20-T vector (TaKaRa, Ohtsu, Japan), sent 15 individual clones for sequencing, and analysed the mutations. To analyse mutation types of T1 and T2 lines, the PCR products were sequenced. The primers used for amplification are shown in Table S3, see online supplementary material. To identify Cas9, the PCR products were amplified using primers specific for Cas9 (Table S4, see online supplementary material).

To analyse off-target mutations, we used CRISPR-P to predict the potential off-target sites (Table S1, see online supplementary material). The corresponding primers (Table S5, see online supplementary material) for each site were used for PCR amplification
the leaves of indicated recombinant pairs, stranded for 3–5 h and injected into the indicated vectors were cultured, suspended, mixed for the SlWRKY45. The released SlWRKY45 represses degradation of SlJAZs through the SCFCOI1 complex to release SlWRKY45 to repress the regulation of downstream genes. JA-Ile triggers vector, respectively [48].

material), and cloned into the pCAMBIA-nLUC or pCAMBIA-cLUC with primers listed in Table S2 (see online supplementary material). SlJAZ11, SlJAZ7NT, SlJAZ11NT, and SlWRKY45 were amplified from the corresponding RNA samples and cloned into the pROK2 vector via the Sma I and Sac I sites to generate the constructs myc-SlJAZ7, myc-SlJAZ10, and myc-SlJAZ11. These vectors were respectively transformed into Agrobacterium strain (GV3101), and infiltrated into leaves of N. benthamiana. At 50 h later, 3 g of the corresponding leaves transiently expressing myc-SlJAZ7, myc-SlJAZ10, or myc-SlJAZ11 were harvested to extract total proteins using RB buffer (25 mM imidazole, protease inhibitor cocktail, 100 mM NaCl, 20 mM 2-mercaptoethanol, 10% glycerol, 0.1% Tween 20, and 50 mM Tris–HCl, pH 7.8).

Pull-down assays

The CDSs of SIWRKY45, SIJAZ7, SIJAZ10, and SIJAZ11 were fused into the pMAL-c5X vector (NEB, CA, USA) via the Sal I and EcoR I sites for MBP fusion. Escherichia coli strains Transetta (DE3) containing the MBP-fused SIWRKY45 vector or empty vector were cultured at 16°C overnight in L broth liquid medium with 0.3 mM IPTG to induce the expression of the corresponding proteins. Amylose resin (NEB, MA, USA) was used to purify these proteins.

The SIJAZ7, SIJAZ10, and SIJAZ11 were inserted into the pROK2 vector via the Sma I and Sac I sites to generate the constructs myc-SIJAZ7, myc-SIJAZ10, and myc-SIJAZ11. These vectors were respectively transformed into Agrobacterium strain (GV3101), and infiltrated into leaves of N. benthamiana. At 50 h later, 3 g of the corresponding leaves transiently expressing myc-SIJAZ7, myc-SIJAZ10, or myc-SIJAZ11 were harvested to extract total proteins using RB buffer (25 mM imidazole, protease inhibitor cocktail, 100 mM NaCl, 20 mM 2-mercaptoethanol, 10% glycerol, 0.1% Tween 20, and 50 mM Tris–HCl, pH 7.8).

Pull-down assays were adopted using previously described methods [49] with slight modification. MBP-fused SIWRKY45 and MBP proteins were respectively added to the amylose resin for 4 h at 4°C, after which 200 μL myc-SIJAZ7, myc-SIJAZ10, or myc-SIJAZ11 protein was added and incubated for 2 h at 4°C. After washing four to six times, the samples were boiled with 100 μL SDS loading buffer at 95°C for 10 min. The proteins were separated via 10% SDS-PAGE for 2 h, and then transferred to PVDF membranes (Millipore, MA, USA). The PVDF membranes were blocked using PBS buffer with 5% nonfat milk, and subsequently incubated with an anti-myc antibody (Abmart, Shanghai, China; 1:5000 dilutions) for 1 h and a goat anti-mouse secondary antibody (Abmart, Shanghai, China; 1:3000 dilutions) for 1 h.

The proteins were observed using enhanced chemiluminescence (ECL) (Solarbio, Beijing, China) in line with the manufacturer’s instructions. Yeast transformation and analysis of protein interactions were carried 0ut in line with the manufacturer’s instructions (Clontech, CA, USA). All Y2H experiments were repeated three biological times.

**Quantitative real-time PCR**

RNA isolation and cDNA synthesis were respectively conducted using kits (DP432, Tiangen, Beijing, China, and AT311–02, Transgen, Beijing, China) in line with the manufacturer’s instructions. qRT-PCR using SYBR Green Mix (Takara, Ohtsu, Japan) with specific primers (Table S6, see online supplementary material) for the Indigo blue (BamHI) detection levels of genes. The qRT-PCR reaction conditions were as below: 3 min at 95°C, 39 cycles of 15 s at 95°C, 10 s at 56°C, and 15 s for 15 s. The internal control gene was tomato ACTIN2. Values of relative gene expression were analysed using the 2^−ΔΔCt method [50]. The qRT-PCR experiments were repeated three biological times.

**Subcellular localization**

SIWRKY45 was in fusion with GFP in the pEGAD vector through the EorI I and BamH I sites to produce the GFP-SIWRKY45 construct. SIJAZ7 and SIJAZ11 (without a stop codon) were cloned

**Yeast two-hybrid screening and yeast two-hybrid assays**

SIJAZ11 was ligated to the pLexA vector with BD, and the cDNA library generated with RKN-infected tomato was used for yeast two-hybrid (Y2H) screening. Y2H screening was carried out based on the manufacturer’s instructions (Clontech, CA, USA). For Y2H assays, full-length CDSs or fragments of SIJAZ1, SIJAZ2, SIJAZ3, SIJAZ4, SIJAZ5, SIJAZ6, SIJAZ7, SIJAZ8, SIJAZ9, SIJAZ10, SIJAZ11, and SIWRKY45 were fused with the pLexA or pB42AD vector. Yeast transformation and analysis of protein interactions were carried out in line with the manufacturer’s instructions (Clontech, CA, USA). All Y2H experiments were repeated three biological times.

**LCI assays**

The CDSs of SIJAZ1, SIJAZ2, SIJAZ3, SIJAZ4, SIJAZ7, SIJAZ10, SIJAZ11, SIJAZ7NT, SIJAZ11NT, and SIWRKY45 were amplified with primers listed in Table S2 (see online supplementary material), and cloned into the pCAMBIA-nLUC or pCAMBIA-cLUC vector, respectively [48]. Agrobacterium strains (GV3101) carrying the indicated vectors were cultured, suspended, mixed for the indicated recombinant pairs, stranded for 3–5 h and injected into the leaves of N. benthamiana. 50 h later, a luciferin solution (0.1 mM luciferin, 0.1% Tween 20, 1 mM NaOH) was sprayed onto the leaves. Photos were obtained by a Tanon 5200 Multi instrument (Tanon, Shanghai, China). The LCI assays were repeated three biological times.

**BiFC assays**

The CDSs of SIWRKY45, SIJAZ7, SIJAZ10, and SIJAZ11 were fused into the cYFP or nYFP vector [49]. Agrobacterium GV3101 carrying the indicated construct pairs was injected into leaves of N. benthamiana. At 50 h after injection, YFP signals were captured using a confocal microscope (TCS-SP5, Leica, Wetzlar, Germany). BiFC assays were repeated three biological times. The primers are shown in Table S2, see online supplementary material.
into the Super1300-GFP vector via the Hind III and Spe I sites, which generated the SlJAZ7-GFP and SlJAZ11-GFP vectors, respectively. These constructs and the corresponding empty vectors were expressed in N. benthamiana leaves through Agrobacterium strain GV3101-mediated expression. GFP signals were captured after 50 h of infiltration with a confocal microscope (TCS-SP5, Leica, Wetzlar, Germany). The primers used in this experiment are shown in Table S2, see online supplementary material. These experiments were repeated three biological times.

**Meloidogyne incognita inoculation assays**

T3 homozygous SIWRKY45-overexpressing plants, Cas9-free T2 homozygous slwrky45 mutants, and their CM wild type plants were used for the experiments. Eggs of *M. incognita* were obtained from the infected tomato roots according to previously described methods [51], and incubated at 28 °C to hatch J2s. When tomato seedlings grew to four true leaves, each plant was inoculated with approximately 400 J2s. At 7 d and 35 d after infection, the roots were soaked with 1.5% sodium hypochlorite for 5 min, washed with water twice, and stained by 3.5% acid fuchsin with 25% acetic acid. After staining, the roots were washed with water and decolorized with the solution of glycerol, acetic acid, and H2O (1:1:1). Then, we weighed the root, and counted the number of galls on each. For counting the egg numbers, nematode eggs of each root were collected according to previously described methods [51], and the egg numbers were counted under a microscope (SMZ-140, Motic, Guangdong, China) in 20 aliquots of 10 μL. After incubation with or without anti-flag-tag antibody (Agarose Conjugated) (Abmart, Shanghai, China) for 4 h at 4 °C, the harvested samples were soaked with 1% Triton X-100, 5 M NaCl and incubated at 65 °C overnight. Finally, DNA was extracted using a DNA extraction kit (GeneBette, Beijing, China). qPCR analysis was used to measure enrichment of promoter fragments by the % input method [54]. A fragment of the SlACTIN2 promoter served as a negative control. The primers used for the experiment are listed in Table S6, see online supplementary material. These experiments were repeated three biological times.

**Dual-LUC assays**

SIWRKY45, SlJAZ11, and GFP were ligated to the pGreenII 62-SK vector [55] via the BamH I and EcoR I sites. The ~1300 bp promoter of SIAOC was fused to the pGREENII 0800 LUC vector [55] via the BamH I and EcoR I sites. Agrobacterium strains GV3101 (pSoup) carrying the corresponding constructs were cultured overnight, combined with the indicated recombinant pairs, incubated for 3–5 h, and co-infiltrated into *N. benthamiana* leaves. At 50 h after infiltration, LUC and REN activities were assessed with a dual-luciferase assay kit (E1910, Promega, WI, USA). The primers used for Dual-LUC assays are shown in Table S2, see online supplementary material. These experiments were repeated three biological times.

**Accession numbers**

The accession numbers for genes are as follows: SIWRKY45 (Solyc08g067360), SIAOC (Solyc02g085730), SILOXD (Solyc03g122340), SlJAZ1 (Solyc07g042170), SlJAZ2 (Solyc12g09220), SlJAZ3 (Solyc03g122190), SlJAZ4 (Solyc12g049400), SlJAZ5 (Solyc03g118540), SlJAZ6 (Solyc01g005440), SlJAZ7 (Solyc11g011030), SlJAZ8 (Solyc06g068930), SlJAZ9 (Solyc08g036640), SlJAZ10 (Solyc08g036620), SlJAZ11 (Solyc08g036660), SlPDF (Solyc07g006380), SlJAZ1 (Solyc11g005330).

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**Data availability**

All data generated in this study are available upon request.

**Conflict of interest**

The authors declare no conflicts of interest.

**Supplementary data**

Supplementary data is available at Horticulture Research online.

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