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Research Paper

SlEAD1, an EAR motif-containing ABA down-regulated novel transcription repressor regulates ABA response in tomato

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ABSTRACT

EAR motif-containing proteins are able to repress gene expression, therefore play important roles in regulating plants growth and development, plant response to environmental stimuli, as well as plant hormone signal transduction. ABA is a plant hormone that regulates abiotic stress tolerance in plants via signal transduction. ABA signaling via the PYR1/PYLs/RCARs receptors, the PP2Cs phosphatases, and SnRK2s protein kinases activates the ABF/AREB/ABI5-type bZIP transcription factors, resulting in the activation/repression of ABA response genes. However, functions of many ABA response genes remained largely unknown. We report here the identification of the ABA-responsive gene *SlEAD1* (*Solanum lycopersicum EAR motif-containing ABA down-regulated 1*) as a novel EAR motif-containing transcription repressor gene in tomato. We found that the expression of *SlEAD1* was down-regulated by ABA treatment, and SlEAD1 repressed reporter gene expression in transfected protoplasts. By using CRISPR gene editing, we generated transgene-free *slead1* mutants and found that the mutants produced short roots. By using seed germination and root elongation assays, we examined ABA response of the *slead1* mutants and found that ABA sensitivity in the mutants was increased. By using qRT-PCR, we further show that the expression of some of the ABA biosynthesis and signaling component genes were increased in the *slead1* mutants. Taken together, our results suggest that *SlEAD1* is an ABA response gene, that SlEAD1 is a novel EAR motif-containing transcription repressor, and that SlEAD1 negatively regulates ABA responses in tomato possibly by repressing the expression of some ABA biosynthesis and signaling genes.

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Introduction

The EAR (ERF-associated amphiphilic repression) motifs, which contain a conserved sequence pattern of (L/F)DLN(L/F)xP, were initially identified in the class II ERFs (Ethylene-responsive factors) and some C2H2 family proteins, and have been shown to confer transcriptional repression activities.[1] Based on sequence comparison of the EAR motifs in class II ERFs, C2H2 family proteins, and some other EAR motif-containing proteins, the consensus sequence patterns of EAR motifs were further refined as LxLxL and DLNxxP.[2] EAR motif-proteins mediated transcriptional repression has been considered to be the main form of transcriptional repression in plants,[3] and a genome wide searching of LxLxL or DLNxxP sequence-containing proteins have identified more than 20,000 EAR motif-containing proteins from 71 different plant species.[4]

EAR motif-containing proteins are able to function as transcription repressors or recruit co-repressors to repress gene expression, therefore play important roles in regulating plants growth and development, as well as plant response to environmental stimuli.[4] For example, KIX8 (KINASE-INDUCIBLE DOMAIN INTERACTING 8) and KIX9 repress leaf growth via function as adaptor proteins for the co-repressor TOPLESS,[5] OFP1 (Ovate family protein 1) regulates cell elongation via repressing the expression of *GA20ox1* (*Gibberellin 20-oxidase 1*),[6] TOE1 (TARGET OF EARLY ACTIVATION TAGGED (EAT) 1) and TOE2 regulate flowering via repressing the expression of *FT* (*FLOWERING LOCUS T*),[7] whereas several repressor ERFs such as ERF4 and ERF7 are involved

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in the regulation of plant responses to stresses.[1,8,9] Several tomato ERFs have also been found to regulate plant growth and development including stomatal density, photosynthesis and fruit ripening, and plant resistance pathogen infections.[10–12]

EAR motif-containing proteins are also involved in the regulation of hormone signaling, for example, ERFs regulate ethylene signaling,[1] JAZ (Jasmonate ZIM domain) proteins regulate jasmonic acid signaling, Aux/IAA proteins regulate auxin signaling, and D53 (DWARF 53) and SMXL7 (SMAX1-LIKE 7) regulate strigolactone signaling.[2,13-16] At least in some cases, changes in hormone singling caused by EAR motif-containing proteins lead to alternation in plants growth and development, as well as plant response to environmental stimuli. For example, D53 and SMXL7 are involved in the regulation of shoot development.[15,16] Whereas AITRs (ABA-induced transcription repressors), a novel family of transcription repressors involved in the regulation of ABA (Abscisic acid) signaling, are able to regulate plant response to abiotic stresses.[17]

ABA is one of the five classic hormones in plants and plays a key role in regulating plant responses to abiotic stresses such as drought, heat, cold, and salinity.[18–20] ABA regulates plant abiotic stress responses via signal transduction.[18–22] ABA signaling is started by the recognition of ABA molecules by the PYR1 (Pyrabactin resistance 1)/PYLs (PYR1-likes) /RCARs (Regulatory component of ABA receptors) receptors.[23–25] Binding of ABA by the PYR/PYLs/RCARs receptors enables their interaction with the A-group PP2Cs (PROTEIN PHOSPHATASE 2Cs) phosphatases,[26,27] who are interacted, at the absence of ABA, with the SnRK2s (NONFERMENTING 1 (SNF1)-RELATED PROTEIN KINASES 2s) protein kinases and inhibiting their activities.[28] Interaction of PYR/PYLs/RCARs with PP2Cs led to the release of SnRK2s, which in turn is able to activate the downstream ABF/AREB/ABI5-type bZIP (basic region leucine zipper) transcription factors,[29,30] resulting in the activation/repression of ABA response genes, and therefore the changes of plant responses to abiotic stresses.[22,26-28,31]

Among the ABA response genes, some have been identified as transcription factor genes from several different families such as the R2R3 MYB family, the NAC (NAM, ATAF1/2, and CUC) family, the bHLH (basic Helix-Loop-Helix) family, the GARP (Golden2, ARR-B, Psr1) family, and the WDR (WD40-repeat) family.[31–39] As mentioned above, we have previously identified a novel family of transcription factors AITRs, we found that AITRs are conserved in angiosperms, and function as negative regulators of ABA signaling.[17] However, even though some of the AITRs contain a full conserved LxLxL EAR motif,[17] AITRs were not identified in the previously genomes wide searching for EAR motif-containing proteins,[4] possibly due to the amino acid sequence diversity of the EAR motif-containing proteins.[4] This suggests that there may still be more EAR motif-containing proteins remained unidentified.

To identify novel EAR motif-containing regulators in ABA signaling in tomato, we performed data mining by using available transcriptome datasets to identify ABA-responsive genes with unknown functions, and then searched for the presence of EAR motif in the candidates. We found that the expression of *SlEAD1* (*Solanum lycopersicum EAR motif-containing ABA down-regulated 1*) was down-regulated by ABA treatment, and SlEAD1 is a novel EAR motif-containing transcription repressor in tomato. By generating and characterizing gene edited *slead1* mutants, we found that SlEAD1 is involved in the regulation of root elongation, and functions as negative regulator in regulating ABA responses in tomato.

Materials and Methods

Plant Materials and Growth Conditions

The Columbia-0 (Col) wild type Arabidopsis (*Arabidopsis thaliana*) was used for protoplast isolation. The Micro-Tom wild type tomato (*Solanum lycopersicum*) was used for protoplast isolation and plant transformation, and as a control for seed germination and root elongation assays. The *slead1* mutants were generated by using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) gene editing in the Micro-Tom wild type background.

To generate seedlings for plant transformation and protoplast isolation, seeds of Micro-Tom wild type tomato were surface-sterilized and germinated as described previously.[40] For seed

germination and root elongation assays, surface-sterilized seeds of the Micro-Tom wild type and the *slead1* mutants were plated on 1/2 MS plates with 0.8% agar and 1% sucrose. The plates were kept at 4°C in darkness for 2 days and then transferred into a plant growth chamber.

For protoplast isolation from Arabidopsis, seeds of the Col wild type were sown directly into soil pots and grew in a growth chamber. The growth conditions in the growth chamber were set at 22°C for Arabidopsis and 24°C for tomato, with a light density of ~120 μmol m^{-2} s^{-1}, and a 16 h/8 h light/dark cycle.

Bioinformatics Analysis of EADs

Homologues of SlEAD1 in other plants were identified by using "Protein Homologs" on phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#), and check on PlantEAR (http://structuralbiology.cau.edu.cn/plantEAR) to see if they have been identified as EAR motif-containing proteins. The full-length amino acid sequences of EADs (Supplemental file 1) were used for phylogenetic analysis by using "One Click" mode on phylogeny (http://www.phylogeny.fr/simple_phylogeny.cgi) with default settings, and for sequence alignment by using BioEdit.

ABA Treatment, RNA Isolation and Quantitative RT-PCR (QRT-PCR)

To examine the expression of *SlEAD1* in response to ABA, 14-day-old Micro-Tom wild type seedlings were cut into pieces and treated with 50 μM ABA or solvent methanol as a control for 4 h in darkness on a shaker at 40 rpm. Samples were frozen in liquid N2 and used for RNA isolation. To examine the expression of ABA signaling and biosynthesis genes in the Micro-Tom wild type and the *slead1* mutants, 14-day-old Micro-Tom wild type and *slead1* mutant seedlings were frozen in liquid N2 and used for RNA isolation.

Total RNA from tomato seedlings were isolated by using the Plant RNA Kit (OMEGA), cDNA was synthesized as described previously,[37] and used as templates for qRT-PCR to examine the expression of *SlEAD1*, and ABA signaling and biosynthesis genes. The primers used for qRT-PCR analysis of *SlEAD1* are 5′-CATCGTGCTAGTGGTTCCCC-3′ and 5′-ATCATCACCACCAAAGAGCGA-3′. The primers used for qRT-PCR analysis of *SlACT2* (*Solyc11g005330*) are 5′-TGGATCTTGCTGGTC GTGATTTA-3′ and 5′-AATTTCCCGTTCAG CAGTGGT-3′. The primers used for qRT-PCR analysis of *SlSnRK2.1* (*Solyc5g056550*), *SlSnRK2.4* (*Solyc02g090390*), and *SlNCED2* (*Solyc08g016720*) have been described previously.[41,42]

The EazyScript First-Strand DNA Synthesis Super Mix (TransGen Biotech) used for cDNA synthesis are able to remove DNA in the RNA sample, and synthesized cDNA were further examined by PCR amplification of *SlACT2* by using intron-spanning primers as described previously,[43] to further ensure there is no DNA contamination. TB Green® Premix EX Taq™ (Takara) were used for qRT-PCR analysis on StepOnePlus (Thermo Fisher Scientific) following a procedure described previously .[44]

Constructs

The construct *NLS-RFP* was used as a nuclear indicator.[45] The reporter construct *LexA-Gal4:GUS*, the activator construct *LD-VP* and the control effector construct *GD* were used for protoplast transfection to examine transcription repressor activities.[6,46]

To generate *GD-SlEAD1* and *GFP-SlEAD1* constructs for protoplast transient transfection, the full length open reading frame (ORF) sequence of *SlEAD1* was amplified by RT-PCR using RNA isolated from 14-day-old Micro-Tom wild type seedlings, double digested with *NdeI* and *SacI* enzymes, and then cloned into digested *pUC19* vector with an N-terminal GD and GFP tag, respectively, under the control of the *CaMV 35S* promoter.[6,46]

To generate gene editing CRISPR/Cas9 constructs of *SlEAD1*, genomic sequence of *SlEAD1* (contains a single exon) was subjected to CRISPRscan (http://www.crisprscan.org) for potential target sequences identification, and selected target sequences were evaluated on Cas-OFFinder (http://www.rgenome.net/cas-offinder/) for potential off-targets. The specific target

sequences selected were 5′-CGCGGCCTTAT TAGCGGTGG(TGG)-3′ and 5′-GCCACACGCG GCCTTATTAG(CGG)-3′. The first sequence was used to generate CRISPR/Cas9 construct by using the *pHDE* vector and following the procedure described by Gao et al.,[47] and both sequences were used to generate CRISPR/Cas9 construct by using the *pHEE401E* vector and following the procedure described by Wang et al.[48] The primers used to generate *pHDE-SlEAD1* construct were 5′-CGCGGCCTTATTAGCGGTGGGTTTTAGAGC TAGAAATAGCAAGTTA-3′ and 5′-CCACC GCTAATAAGGCCGCGAATCACTACTTCGAC-TCTAGC-3′. The primers used to generate *pHEE-SlEAD1* construct were *SlEAD1-DT1-BsF*, 5′-ATATATGGTCTCGATTGGCGGCCTTATTAG-CGGTGGGTT-3′, *SlEAD1-DT1-F0*, 5′-TGGCGGCCTTATTAGCGGTGGGTTTTAGAG-CTAGAAATAGC-3′, *SlEAD1-DT2-R0*, 5′-AACCTAATAAGGCCGCGTGTGGCAATCTCT-TAGTCGACTCTAC-3′ and *SlEAD1-DT2-BsR*, 5′-ATTATTGGTCTCGAAACCTAATAAG GCCGCGTGTGGC-3′. The primers *pER8-U95-F* and *E9-U29-R*, and *U626-IDF* and *U629-IDR* described previously [47-49]) were used for colony PCR and sequencing of *pHDE-SlEAD1* and *pHEE401E-SlEAD1* constructs, respectively.

Plant Transformation, Transgenic Plant Selection and Cas9-free Mutant Isolation

Cotyledons and hypocotyls were collected from ~10-day-old Micro-Tom wild type seedlings and transformed with the CRISPR/Cas9 constructs via agrobacterium-mediated transformation, by using the plant tissue culture method as described previously.[40] Transgenic T1 plants were identified by examining the present of *Cas9* in the regenerated plants. Gene editing status in confirmed T1 transgenic plants was examined by amplifying and sequencing the genomic sequence of *SlEAD1*. T2 seeds were collected from gene edited T1 plants, germinated and grew in soil pots, and used to identify homozygous *Cas9*-free mutant by PCR amplification and sequencing.

DNA Isolation and PCR

Genomic DNA was isolated as described previously.[50] To identify T1 transgenic plants, DNA was isolated from leaves of regenerated plants and used for PCR amplification of *Cas9* fragment. To identify gene edited mutants for *SlEAD1*, DNA was isolated from leaves of the confirmed T1 transgenic plants and used for PCR amplification and sequencing of *SlEAD1*. To isolate homozygous *Cas9*-free mutants, DNA was isolated from leaves of T2 plants germinated from the confirmed mutants in T1 generation, and used for PCR amplification of *Cas9* fragment, as well as amplification and sequencing of *SlEAD1*. The primers for amplifying *Cas9* in *pHDE-SlEAD1* transformed plants are 5′-GACAAGAAGTAC TCCATTGGG-3′ and 5′-CAAACAGGCCGTTC TTCTTC-3′, and in *pHEE-SlEAD1* transformed plants are as described previously.[49]

Plasmid DNA Isolation, Protoplast Isolation and Transfection

Plasmid DNA of the reporter and effector were isolated by using Endo-Free Plasmid Maxi Kit (OMEGA). Arabidopsis protoplasts were isolated from rosette leaves of 3- to 4-week-old Col wild type plants as described previously.[6,17,51] Tomato protoplasts were isolated by using the procedure for Arabidopsis protoplast isolation as described previously,[51] except that cotyledons of 8- to 10-day-old Micro-Tom wild type plants were used for protoplast isolation, and the time for enzyme digestion increased from ~3 h to 6–8 h.

For subcellular location assay in Arabidopsis and tomato protoplasts, plasmids of *GFP-SlEAD1* and *NLS-RFP* were co-transfected into protoplasts. After 20–22 h incubation in darkness, GFP and RFP florescence in the transfected protoplasts were observed under a fluorescence microscope.

For transcriptional activity assay in Arabidopsis and tomato protoplasts, plasmids of the reporter gene *LexA-Gal4:GUS*, the activator gene *LD-VP* and the effector gene *GD-SlEAD1* were co-

transfected into protoplasts, the effector gene *GD* was co-transfected as a control. After 20–22 h incubation in darkness, GUS activities in the transfected protoplasts were measured by using a microplate reader.

ABA Sensitivity Assays

ABA inhibited seed germination and root elongation assays were performed as described previously with some modifications.[52–54]

For ABA inhibited seed germination, seeds of the Micro-Tom wild type and the *slead1* mutants were surface-sterilized and plated on 1/2 MS plates containing 2.5 μM ABA or without ABA as a control. Pictures were taken 4 d after the plated were transferred into the growth chamber, the number of germination seeds was counted, and the germination rate was calculated. Thirty seeds for each genotype were used for the experiment, and the experiment was repeated three times.

For primary root length and ABA inhibited root elongation assays, seeds of the Micro-Tom and the *slead1* mutants were surface-sterilized and plated on 1/2 MS plates. After 3 d, germinated seeds were chosen and transferred to 1/2 MS plates with 0, 5, and 10 μM ABA and grown vertically. Then, pictures were taken 6 d after the transfer, root length was measured, and percentage of inhibition was calculated. At least 10 seedlings for each genotype were used for the experiment, and the experiment was repeated three times.

Results

Expression of SlEAD1 Is Down-regulated by ABA Treatment

In an attempt to identify novel players in ABA signaling in tomato, we performed data mining by using available transcriptome datasets, we found that an unknown function gene *SlEAD1* (*Solyc12g099500*) was identified as a differential expressed gene in response to ABA during tomato fruit ripening,[55] but it is expression level remained largely unchanged in tomato leaves 24 h after the spray of ABA.[56]

Considering that ABA response genes response to ABA in hours or even minutes, we examined the expression of *SlEAD1* in tomato seedlings treated with ABA for 4 h. Quantitative RT-PCR analysis shows that the expression level of *SlEAD1* reduced ~2 folds in the ABA treated seedlings (Fig. 1) suggest that *SlEAD1* is ABA response gene.

Amino acid sequence BLASTing on NCBI (https://blast.ncbi.nlm.nih.gov) indicates that EADs are plant specific proteins, and are likely presented only in the flowering plants. However, considering that some genome databases are very preliminary, we could not rule out the possibility that EADs may also present in other plants. Phylogenetic analysis of SlEAD1 and some

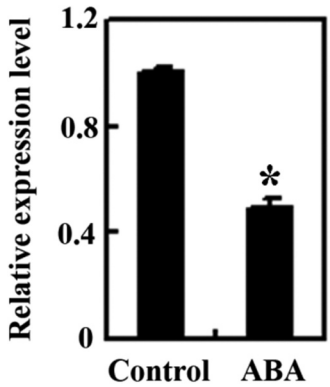

Figure 1. Expression of *SlEAD1* in response to ABA treatment. The expression of *SlEAD1* was down-regulated by ABA treatment. Fourteen-day-old Micro-Tom wild type seedlings were cut into pieces and treated in darkness for 4 h with 50 μM ABA or solvent methanol as a control. Samples were frozen in liquid N_2, and total RNA was isolated and used for cDNA synthesis. The synthesized cDNA was used as template for qRT-PCR to examine the expression of *SlEAD1*, and the expression of *SlACT2* was examined and used as an inner control. The expression level of *SlEAD1* in mock-treated samples was set as 1, and its relative expression level in ABA treated samples was calculated. Data represent the mean ± SD of three replicates. *Significantly different from that in the mock-treated samples ($p < .001$).

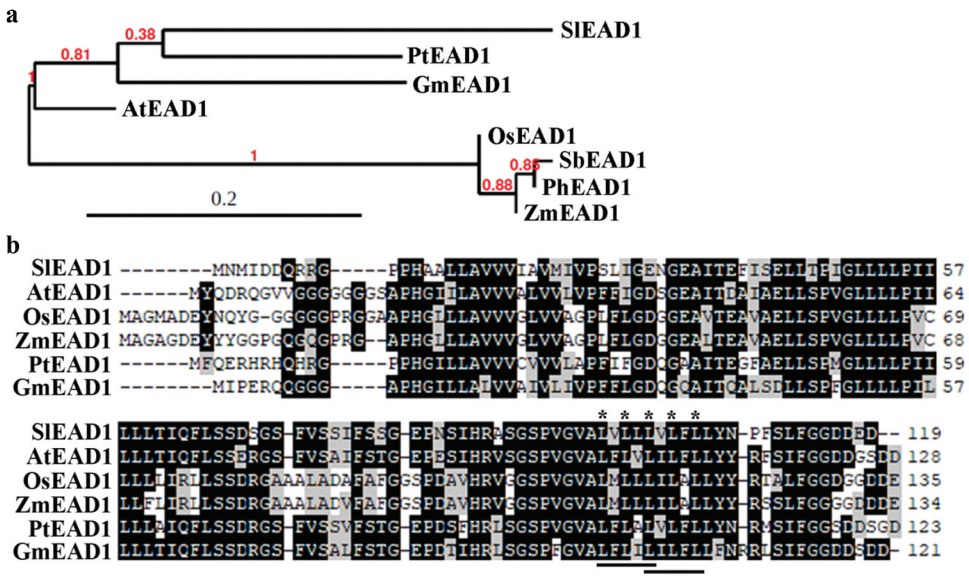

Figure 2. Phylogenetic analysis and amino acid alignment of EADs in different plants. (a) Phylogenetic analysis of SlEAD1 and EADs in several other plant species. EADs in other plants were identified on phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#), and their full-length amino acid sequence were used for phylogenetic analysis by using "One Click" mode on phylogeny (http://www.phylogeny.fr/simple_phylogeny.cgi) with default settings. (b) Amino acid sequence alignment of EADs. Full-length amino acid sequences of EADs were used for sequence alignment by using BioEdit. The identical amino acids were shaded in black, and the similar ones in gray. Underlines indicate the overlapped LxLxL EAR motifs, and stars indicate the conserved L residues.

representative EADs from several selected plants including the model plant Arabidopsis, the tree poplar, the crops soybean, rice, sorghum and corn, and the grass *Panicum hallii* indicates that EADs from dicots formed a clade, whereas that from monocots formed another clade (Fig. 2a). Yet amino acids in the representative EADs are highly conserved (Fig. 2b). In addition, an LxLxL type EAR repressor motif initial found in repressor ERFs was presented in SlEAD1 (Fig. 2b); therefore, it was named *Solanum lycopersicum* EAR motif-containing ABA down-regulated 1. We also found that there are actually two overlapped LxLxL type EAR motifs in all the EADs examined (Fig. 2b), similar to that in the chimeric repressor SRDX.[57]

SlEAD1 Is a Transcription Repressor

Considering that the EAR motif is found in, and is required, at least partially for the repression function of transcription repressors including the repressor ERFs, the Aux/IAA proteins, the OFPs, and the AITRs,[1,6,14,17] we assume that SlEAD1 may also functions as a transcription repressor.

To examine if that is the case, we first observed the subcellular localization of SlEAD1 by using protoplast transit transfection assays. Plasmid DNA of *GFP-SlEAD1* was transfected into protoplasts isolated from Arabidopsis leaves, and GFP fluorescence was observed by using a fluorescence microscope. We found that GFP fluorescence was predominantly observed in the nucleus (Fig. 3a). We also transfected plasmid DNA of *GFP-SlEAD1* into protoplasts isolated from tomato cotyledons and found that SlEAD1 was predominantly localized in the nucleus (Fig. 3a).

We then examined transcriptional activities of SlEAD1 in transfected protoplasts. Plasmids of the reporter gene *LexA-Gal4:GUS*, the activator gene *LD-VP* and the effect gene *GD-SlEAD1* or the control gene *GD* were co-transfected into protoplasts isolated from Arabidopsis leaves, and GUS activities in the protoplasts were measured by using a microplate reader. As shown in Fig. 3b, GUS activity activated by the activator LD-VP was repressed by the co-transfection of the effector gene *GD-SlEAD1*. Similarly, Co-transfection of the effector gene *GD-SlEAD1* in protoplasts isolated from tomato cotyledons also resulted in repression of the reporter gene (Fig. 3b). These results indicate that SlEAD1 functions as a transcription repressor.

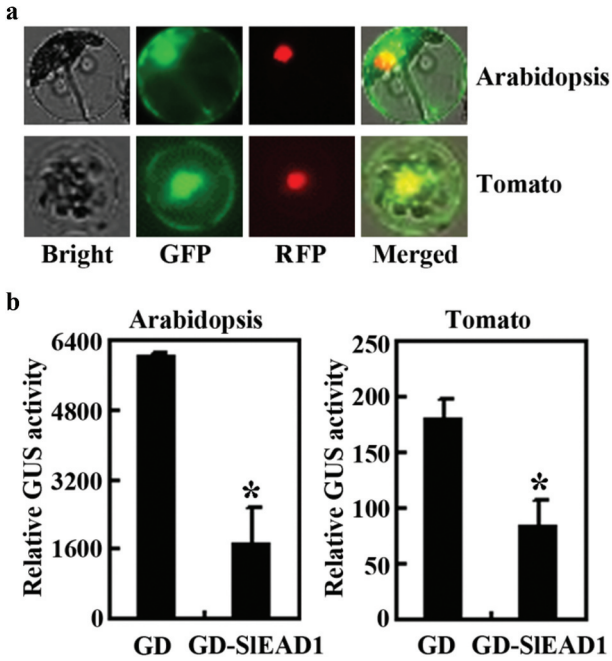

Figure 3. SlEAD1 is a transcription repressor. (a) Subcellular localization of SlEAD1. Plasmids of the effector gene *GFP-SlEAD1* and *NLS-RFP* were co-transfected into protoplasts isolated from Arabidopsis leaves and tomato cotyledons, respectively. After transfection, the protoplasts were incubated at room temperature in darkness for 20~22 h, then the GFP and RFP fluorescence was observed under a confocal microscope. (b) Transcriptional activities of SlEAD1. Plasmids of the reporter gene *LexA-Gal4:GUS*, the transcription activator gene *LD-VP* and the effector gene *GD-SlEAD1* were co-transfected into protoplasts isolated from Arabidopsis leaves and tomato cotyledons, respectively. Co-transfection of the effector gene *GD* was used as a control. After transfection, the protoplasts were incubated at room temperature in darkness for 20~22 h, then GUS activities were measured by using amicroplate reader. Data represent the mean ± SD of three replicates. *Significantly different from that of the GD control ($p < .001$).

The Gene Editing Slead1 Mutants are Hypersensitive to ABA

Having shown that the expression of *SlEAD1* was suppressed by ABA and SlEAD1 is as a transcription repressor, we wanted to further examine the function of SlEAD1 in regulating ABA response in tomato.

To do that, we decided to generate mutants by using CRISPR/Cas9 gene editing, since it has been able to generate transgene-free mutants in the model plant Arabidopsis as well as crops.[47,58-60] Two CRISPR/Cas9 constructs, *pHDE-SlEAD1* and *pHEE-SlEAD1* targeting one and two sites, respectively (Fig. 4a,b), were generated and used for tomato plant transformation. Gene editing was observed in T1 plants generated with both constructs, and transgene-free mutants were obtained in T2 generations. The *slead1-c1* mutant was generated with *pHDE-SlEAD1* construct, and has a single nucleotide insertion in the target site of *SlEAD1*, whereas the *slead1-c2* mutant was generated with *pHEE-SlEAD1* construct, and has a single nucleotide insertion in one target site, and 5 bp deletion in another target site of *SlEAD1* (Fig. 4c). In both mutants, the nucleotide indels led to a few amino acids substitution and a premature stop in SlEAD1 (Fig. 4d).

By using the mutants obtained, we examined if SlEAD1 may regulate ABA response in tomato plants. In seed germination assays, we found that ABA inhibited seed germination in both the Micro-Tom wild type and the *slead1* mutants (Fig. 5a). However, no different was observed for both the Micro-Tom wild type and the *slead1* mutants in the control plates, they all reached an ~60% generation rate (~20 out of 30) 4 d after the transfer, but that in ABA-contained plates were ~50% (~15 out of 30) and 30% (~9 out of 30), respectively, for the Micro-Tom wild type and the *slead1* mutants (Fig. 5b), indicating that the *slead1* mutants are more sensitivity to ABA treatment.

Similarly, the *slead1* mutants also showed an increased sensitivity to ABA treatments in root elongation assays (Fig. 6). We also noted that

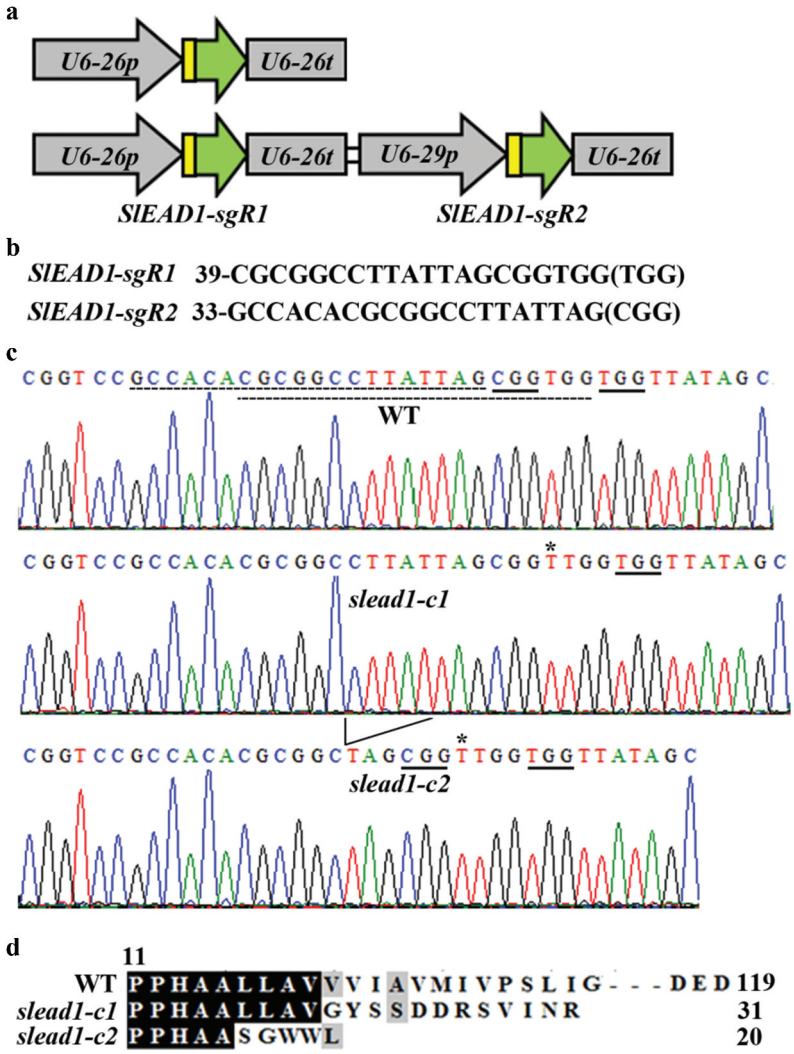

Figure 4. Generation of transgene-free *slead1* mutants. (a) Diagram of the *sgRNA* expression cassettes cloned into the *pHDE-SlEAD1* (up panel) and the *pHEE-SlEAD1* (low panel) vectors, respectively. (b) Target sequences of *SlEAD1*. The numbers indicate the positions of the first nucleotide in the target sequences relative to the first nucleotide in the coding sequence of *SlEAD1*. The NGG PAM sites immediately after the target sequences are in brackets. (c) Editing status of *SlEAD1* in the *slead1* mutants. The *slead1-c1* and *slead1-c2* mutants were obtained by transforming Micro-Tom wild type plants with the *pHDE-SlEAD1* and *pHEE-SlEAD1* constructs, respectively. Editing status of *SlEAD1* in T1 plants was examined, and transgene-free homozygous mutant plants were isolated from T2 generations. Stars indicate the single T nucleotide insertion in the *slead1-c1* and *slead1-c2* mutants, and arrow head indicates the 5 bp deletion in the *slead1-c2* mutant. Solid underlines indicate the PAM sites, and dash underlines indicated the target sequences. (d) Amino acid alignment of SlEAD1 in the Micro-Tom wild type and the *slead1* mutants. ORFs of *SlEAD1* in the *slead1* mutants were identified by using ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/), and predicted amino acid sequences were used for alignment with the amino acid sequence of SlEAD1. Number at the N-terminal indicates the amino acid position relative to the first M amino acid, and the numbers at the C-terminal indicate total amino acid numbers of SlDAE1 in the Micro-Tom wild type and the *slead1* mutants.

the primary root length in the *slead1* mutant seedlings was shorter when compared with the Micro-Tom wild type seedlings (Fig. 6a). Quantitative results show that the root length for 9-day-old Micro-Tom wild type seedlings was ~9 cm, but that for the *slead1* mutants was about ~8 cm (Fig. 6b). Root elongation in the Micro-Tom wild type seedlings was inhibited about ~50% and ~60%, respectively, by 5 μM and 10 μM ABA, where as that for the *slead1* mutant seedlings was ~60% and ~70%, respectively (Fig. 6c).

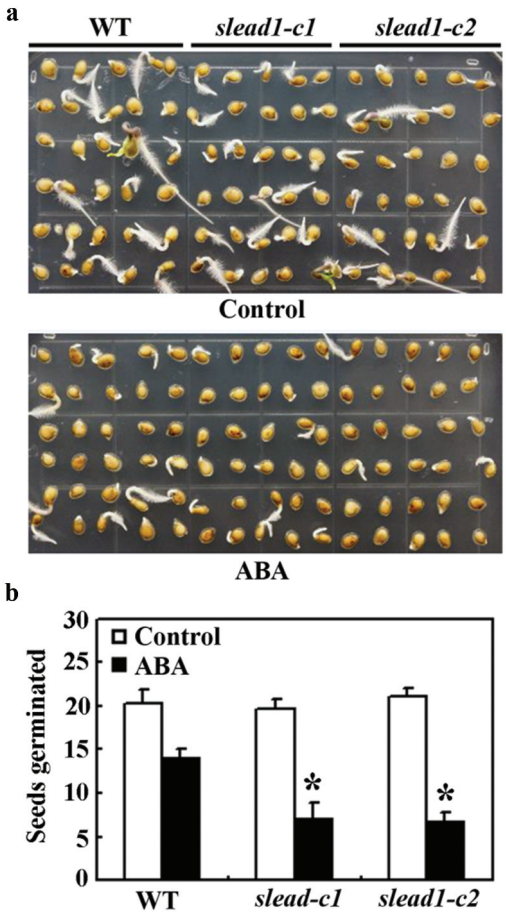

Figure 5. Effects of ABA on seed germination of the Micro-Tom wild type and the *slead1* mutants. (a) Seed germination of the Micro-Tom wild type and the *slead1* mutants in response to ABA treatment. Seeds were sterilized and plated on 1/2 MS plates or plates with 2.5 μM ABA. The plates were kept at 4°C in darkness for 2 d, and then transferred to a growth room. Pictures were taken 4 d after the transfer. (b) Number of seed germinated of the Micro-Tom wild type and the *slead1* mutants in response to ABA treatment. Seeds germinated were counted 4 d after the transfer, and average number of seed germinated was calculated. Data represent the mean ± SD of three replicates. *Significantly different from that of the wild type ($p < .01$).

Expression of ABA Signaling and Biosynthesis Genes Was Affected in the Slead1 Mutants

Changes in ABA sensitivity may caused by alternation in ABA signaling and/or ABA biosynthesis, to examine why ABA sensitivity was increased in the *slead1* mutants, we examined the expression levels of the core ABA signaling regulator genes and ABA biosynthesis genes in the *slead1* mutants. We found that the expression levels of the core ABA signaling regulator genes *SlSnRK2.1* and *SlSnRK2.4*, and the ABA biosynthesis gene *SlNCED2* were increased in the *slead1* mutants (Fig. 7a). These results suggest that SlEAD1 may regulate ABA response in tomato by negatively regulating the expression of ABA signaling genes and ABA biosynthesis genes (Fig. 7b).

Discussion

EAR motif-containing proteins are involved in the regulation of hormone signaling, including auxin signaling, ethylene signaling, jasmonic acid signaling, strigolactone signaling.[1,2,13-16] We previously identified AITRs as a novel family of transcription repressors that are involved in the regulation of ABA signaling, and at least some of the AITRs contain a full conserved LxLxL EAR motif.[17] We identified here SlEAD1 as a novel EAR motif-containing protein that plays a role in regulating ABA response in tomato, and EADs may represent a novel family of transcription repressors in plants.

First, SlEAD1 contains two overlapped LxLxL EAR motifs(Fig. 2b), similar to that in SRDX, a chimeric activate repressor that can convert a

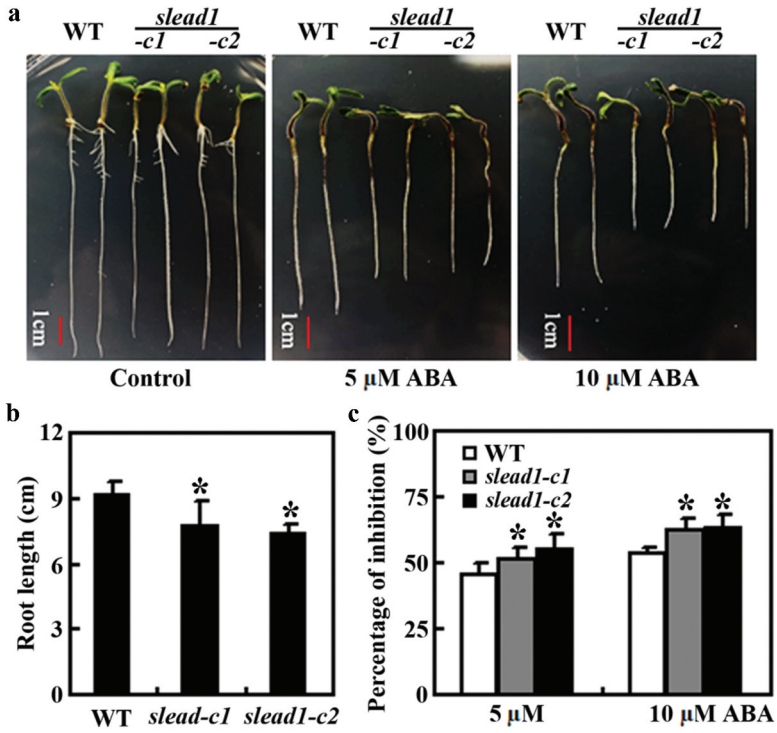

Figure 6. Effects of ABA on root elongation of the Micro-Tom wild type and the *slead1* mutants. (a) Root elongation of the Micro-Tom wild type and the *slead1* mutants in response to ABA treatment. Seeds were sterilized and plated on 1/2 MS plates. The plates were kept at 4°C in darkness for 2 d, and then transferred to a growth room. After 3 d, germinated seeds were transferred to control plates, or plates containing 5 and 10 μM ABA, respectively, and grown vertically for 6 d before the pictures were taken. (b) Root length of Micro-Tom wild type and the *slead1* mutants. Root length of the seedling was measured 6 d after the transfer. Data represent means ± SD of at least 10 seedlings. *Significantly different from that of the wild type ($p < .05$) (c) Percentage of inhibition on root elongation by ABA. Root length of the seedling was measured 6 d after the transfer, and the percentage of inhibition on root elongation was calculated. Data represent means ± SD of at least 10 seedlings. *Significantly different from that of the wild type ($p < .01$).

transcription activator to a repressor,[57] and have been used to study the functions of transcription factors from different families, such as the ERF family 4 Huang et al. 61], the R2R3 MYB family,[62–64] the WRKY family,[65–67] and the NAC family.[68,69] Most importantly, the overlapped LxLxL EAR motifs are conserved on EADs from other plant species such as Arabidopsis, rice, and poplar (Fig. 2). Second, SlEAD1 functions as a transcription repressor, SlEAD1 was found to be predominately localized in nucleus in transfected protoplasts, and consistent with the presence of EAR motifs, SlEAD1 repressed reported gene expression in both Arabidopsis and tomato protoplasts (Fig. 3). Third, ABA sensitivities in *slead1* mutants were increased (Fig. 5, Fig. 6), suggesting that SlEAD1 may play a negative role in regulating plant response to ABA.

EAR motif-containing proteins mediated transcription repression can be achieved by at least two different ways. One is epigenetic modification[3] by recruit a histone deacetylase (HDAC) to form a HDAC complex via interactions with co-suppressors. For example, ERF7 can interact with SIN3, whereas ERF3 and ERF4 can interact with SAP18 (SIN3 ASSOCIATED POLYPEPTIDE P18), to recruit HDA19 to form an HDAC complex.[8,9,70,71] Another is to interfere with the activities of other transcription factors via directly or indirectly binding to them. For example, the Aux/IAA proteins interact with activator ARFs to repress their activities, the JAZ (JASMONATE ZIM) domain proteins interact with MYC activators to repress their activities, whereas OFP1 and OFP4 interact with KNAT7 to enhance its repression activities.[2,13,14,72-75] We found that SlEAD1 is an EAR motif-containing protein (Fig. 2), and

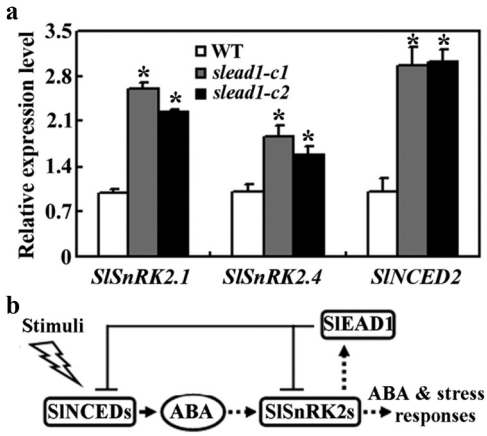

Figure 7. Expression of ABA signaling and biosynthesis genes in the Micro-Tom wild type and the *slead1* mutants. (a) Expression of *SlSnRKs* and *SlNCED2* in the wild type and *slead1* mutant tomato seedlings. Total RNA was isolated from 14-day-old Micro-Tom wild type and *slead1* mutant seedlings and used for cDNA synthesis. The synthesized cDNA was used as template for qRT-PCR to examine the expression of ABA signaling and biosynthesis genes, and the expression of *SlACT2* was examined and used as an inner control. The expression levels of the corresponding genes in the Micro-Tom wild type seedlings were set as 1, and their relative expression levels in *slead1* mutant seedlings were calculated. Data represent the mean ± SD of three replicates. *Significantly different from that in the wild type ($p < .01$). (b) A diagram showing the regulation and roles of SlEAD1 in ABA signaling in tomato.

functions as transcription repressors (Fig. 3), however, it is unclear how SlEAD1 may mediate transcription repression, identification of proteins that can interact with SlEAD1 may enable to figure this out.

ABA signaling via the PYR/PYLs/RCARs receptors, the PP2Cs phosphatases and SnRK2s protein kinases activates the downstream ABF/AREB/ABI5-type bZIP transcription factors, leading to the activation/repression of ABA response genes and plant responses to abiotic stresses.[22,26-28,31] We show that the expression of *SlEAD1* was down-regulated by ABA; therefore, it will be of interest to examine if ABF/AREB/ABI5-type bZIP transcription factors may directly regulate the expression of *SlEAD1*.

The function mechanisms of SlEAD1 are also needed to be further studied. Our data show that ABA sensitivities were increased in the *slead1* mutants (Fig. 5, Fig. 6), and consistent with SlEAD1's transcription repression activities, the expression levels of ABA biosynthesis gene *SlNCED2* and ABA signaling key component genes *SlSnRK2.1* and *SlSnRK2.4* were increased in the *slead1* mutants, indicating that SlEAD1 may play a feedback regulating role in ABA signaling (Fig. 7), it is worthwhile to examine if SlEAD1 is involved in the regulation of plant abiotic stress responses, to examine if these genes are directly targets of SlEAD1, and to examine how SlEAD1 may regulate the expression of these genes, therefore to uncover the functional mechanism of SlEAD1 in regulating ABA signaling and plant response to abiotic stresses.

In summary, we found that SlEAD1 is a novel EAR motif-containing transcription repressor, the expression of *SlEAD1* was down-regulated by ABA, and SlEAD1 negatively regulates ABA responses in tomato.

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Author Contributions

SW conceived the study. SW, WW, and TW designed the experiments. WW, XW, YW, GZ, CW, SH, A, and RL performed the experiments. WW and SW analyzed the data. SW

and WW drafted the manuscript. All the authors participated in the revision of the manuscript.

Declaration Of Interest Statement

The authors declared no conflict of interest.

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