Supplementary Materials for

ZAT10 regulates root thermomorphogenesis via very-long-chain fatty acids signaling

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Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana Col-0 was used as the wild type. Additionally, the T-DNA insertion lines, *kcs1-5* (SALK_200839C), *kcs2* (CS71727), *kcs20* (CS71728), *zat10* (SALK_054092), *myc2-1* (SALK_017005), *sty46-1* (SALK_112195), *sty46-2* (CS887714), *acs6* (CS16569), *mkk9-1* (CS872513), and *mkk9-2* (SALK_017378) were obtained from the SALK collection and the seed stock center of Arabidopsis Biological Resource Center. The *jin1-2*/*myc2* (S1) was provided by Dr. Roberto Solano (Centro Nacional de Biotecnologia-CSIC, Madrid, Spain). Notably, these mutants were genotyped using left-border primers on the T-DNA, right-side primers on the genome, and left-side primers on the genome (Table S1). Right-side and left-side primers were designed on the T-DNA express website (http://signal.salk.edu/tdnaprimers.2.html). Additionally, the *kcs1*/*2*/*20* triple mutants were generated by crossing genome edited *kcs1*/*kcs2* double mutant and *kcs20* homozygous mutants.

All seeds were sterilized with 1% bleach and 0.05% Triton X-100 for 5 min and washed three times with sterilized water. Seeds were germinated on Murashige and Skoog (FUJIFILM Wako Pure Chemical) medium supplemented with 1% sucrose and 1% agarose after 2 days at 4 °C. Plants were grown vertically in a chamber (Panasonic) at 22 °C or 28 °C under a 16-h light/8-h dark cycle. For Cmix treatment, *kcs1*/*2*/*20* mutants were grown on media supplemented with 20 μM of each saturated very-long-chain fatty acids (VLCFAs) with carbon chain lengths of 20, 22, 24, 26, 28, and 30.

Plasmid construction and plant transformation

The *kcs1*/*kcs2* double mutant was generated by genome editing of *KCS1* in the *kcs2* background using the CRISPR-Cas9 system. A guide RNA was designed to target position 286–306 of the *KCS1* coding region, and the corresponding oligonucleotides were cloned into the pKAMA-ITACHI vector following the method described by Tsutsui et al., resulting in the construct pKAMA-ITACHI-*kcs1* (S2).

Genomic DNA from Col-0 was used as the template to amplify 2,736, 2,988, and 2,564 bp upstream regions of *pKCS1*, *pKCS2*, and *pZAT10*, respectively, for promoter cloning. Additionally, the 5' and 3' ends of the *pKCS1*, *pKCS2*, and *pZAT10* PCR amplicon were designed with 20 bp homologous sequences to pENTR5'-*pMYB93* (S3) in the primers. The plasmid vector was generated by amplifying a recombination-ready PCR product from pENTR5'-*pMYB93*, excluding the *pMYB93* region. These PCR products were assembled using NEBuilder® HiFi DNA Assembly Cloning Kit (New England Biolabs) to insert the *pKCS1*, *pKCS2*, and *pZAT10* regions, resulting in the construction of pENTR5'-*pKCS1*, pENTR5'-*pKCS2*, and pENTR5'-*pZAT10*. The cDNA region of *KCS1* and *KCS2* were amplified via PCR using cDNA pool generated from Col-0 root RNA as a template. The genomic region of *ZAT10* was amplified via PCR using Col-0 genome as a template. These amplicons were inserted at the 5' side of *YFP* in the pDONR201-*YFP* plasmid using the NEBuilder® HiFi DNA

Assembly Cloning Kit, resulting in the construction of pDONR201-*KCS1-YFP*, pDONR201-*KCS2-YFP*, and pDONR201-*ZAT10-YFP*.

For *pKCS1::KCS1-YFP*, *pKCS2::KCS2-YFP*, and *pZAT10::ZAT10-YFP*, pENTR5'-*pKCS1*, pENTR5'-*pKCS2*, and pENTR5'-*pZAT10* and pDONR201-*KCS1-YFP*, pDONR201-*KCS2-YFP*, and pDONR201-*ZAT10-YFP* were cloned into R4pGWB501 (S4) using LR Clonase II (Thermo Fisher Scientific). For the *pXVE::ZAT10-YFP* construct, *ZAT10-YFP* containing pDONR201 was cloned into pMDC7 (S5) using LR Clonase II. The resulting plasmids (pKAMA-ITACHI-kcs1, *pKCS1::KCS1-YFP*, *pKCS2::KCS2-YFP*, *pZAT10::ZAT10-YFP,* and *pXVE::ZAT10-YFP*) were transferred into *Agrobacterium tumefaciens* (C58C1 pMP90) cells and transformed into *kcs2*, Col-0, and *zat10* mutants. The list of primers used in this study is provided in Table S1.

Fatty acid (FA) extraction and GC-MS analysis

FA extraction was performed as previously described by Patel et al. (S6) and Uemura et al. (S3), with some modifications. Briefly, Col-0, *kcs1-5*, and *kcs1*/*2*/*20* roots were grown in Murashige and Skoog medium at 22 °C or 28 °C for 13 days. Root samples (200 mg) were collected from each group, frozen, and ground. Notably, the preparation of FA methyl esters (FAME) for GC-MS analysis and the GC-MS procedure were performed following the method described by Uemura et al. (2023(S3)). FAME from each sample was quantified using corresponding standards (C2–C24 even carbon-saturated FAMEs; Sigma-Aldrich).

Phenotypic and microscopic analysis

To measure whole root length, the roots of growing seedings in plates were scanned using a flatbed scanner GT-7400U (Epson). Root length was measured by analyzing the images using ImageJ image-processing software (imagej.net.software/fiji/).

Laser scanning confocal microscopy of Col-0, *kcs1*/*2*/*20*, *zat10*, *myc2-1*, *jin2-1*, *sty46-1*, *sty46-2*, *mkk9-1*, *mkk9-2*, *pZAT10::ZAT10-YFP*, *pMYC2::GFP* roots stained with propidium iodide (PI; FUJIFILM Wako Pure Chemical) was performed using a Leica SP8 system (Leica). Briefly, roots were stained with PI in water (10 μg ml^{-1}) for 3–5 min, with 488 nm excitation and 490–543 nm emission for YFP, 488 nm excitation and 500–550 nm emission for GFP, and 555 nm excitation and 580–680 nm emission for PI. Image assembly and measurements of the lengths of 10 meristematic zone, the elongation zone, and cells in the maturation zone were performed using the LAS X software (Leica).

Gene expression analysis

RNA was isolated from whole roots of 10-day-old seedlings grown at 22 °C or 28 °C using the RNeasy Plant kit (QIAGEN). Thereafter, the extracted RNA was reverse-transcribed to generate first-strand cDNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO Co., Ltd). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed on a real-time PCR

Eco system (PCRmax) using the THUNDERBIRD SYBR qPCR Mix (TOYOBO). Primers used in this study are listed in Table S1. The RT-qPCR efficiency and C_T value were determined using the standard curves for each primer set. Efficiency-corrected transcript values of at least three biological replicates for all samples were used to determine relative expression values. Each value was normalized against the level of *PDF2* (S7).

RNA-seq and data analysis

Briefly, 100 ng of RNA extracted from the root of 10-day-old Col-0 and *kcs1/2/20* plants grown at 22 °C or 28 °C were reverse-transcribed to generated cDNA libraries using the NEBnext Ultra II RNA Library Prep kit (New England Biolabs) following manufacture protocols. Thereafter, both ends of cDNA libraries were sequenced for 60 cycles using a paired-end module on an Illumina NextSeq 500 platform (Illumina). Three biological replicates were conducted for each experiment.

For sequence data analysis, the short-read sequencing results were mapped to the Arabidopsis genome (TAIR10: www.arabidopsis.org/) using the Bowtie software (S8). After normalizing the data, differential expression analysis was performed to determine differentially expressed genes (DEGs) in Col-0 and *kcs1/2/20* grown at 22 °C and 28 °C using edgeR package in R (S9) and the following threshold: FDR of $q < 0.05$. Notably, the RNA-seq data were deposited with links to BioProject accession number PRJDB35754 in the DNA Data Bank of Japan (DDBJ) BioProject database (http://www.ddbj.nig.ac.jp/index-e.html). Gene ontology functional annotation was performed using the Metascape website (S10; https://metascape.org/gp/index.html#/main/step1).

DAP-seq and ChIP-seq reanalysis

DAP sequence reads of ZAT10 were obtained from the NCBI database (GSE60143) and MYC2 ChIP-seq data (CRR032460) were obtained from Genome Sequence Archive (S11). Thereafter, these data were mapped to the *A. thaliana* genome (TAIR10) using Bowtie2 with default parameters (S12; S13). The sequence alignment/map (SAM) files generated using Bowtie2 were converted to binary alignment/map (BAM) format using SAMtools (Li et al., 2009).

DAP-seq peaks were identified by comparing DAP-seq data from Col-0 and ZAT10 using model-based analysis of ChIP-seq (MACS2) with parameters "-p 0.05 -g $1.118e^8$" ($p < 0.05$, peak score > 30) (S14). Peaks were assigned to genes located within 1,000 bp upstream of translation start site using the ChIPpeakAnno package from Bioconductor and the R programming environment.

Statistical analysis

All statistical analysis were conducted using Microsoft Excel or R programs. Details of the analysis are given in the figure legends. Briefly, statistical significance was determined using a Student's *t*-test ($p < 0.05$ or 0.01), Tukey's honestly significant difference (HSD) test ($p < 0.05$), or two-way ANOVA ($p < 0.05$, 0.01, or 0.001).

References

S1. Lorenzo O., Chico J. M., Sánchez-Serrano J. J. & Solano R. JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. *Plant Cell* **16**(7), 1938–1950 (2004).

S2. Tsutsui H. & Higashiyama T. pKAMA-ITACHI vectors for highly efficient CRISPR/Cas9-mediated gene knockout in Arabidopsis thaliana. *Plant Cell Physiol.* **58**(1), 46–56 (2017).

S3. Uemura Y., Kimura S., Ohta T., Suzuki T., Mase K., Kato H. et al. A very long chain fatty acid–responsive transcription factor, MYB93, regulates lateral root development in Arabidopsis. *Plant J.* **115**(5), 1408–1427 (2023).

S4. Nakagawa T., Nakamura S., Tanaka K., Kawamukai M., Suzuki T., Nakamura K. et al. Development of R4 Gateway binary vectors (R4pGWB) enabling high-throughput promoter swapping for plant research. *Biosci. Biotechnol. Biochem.* **72**(2), 624–629 (2008).

S5 Moore, I., Samalova, M. & Kurup, S. Transactivated and chemically inducible gene expression in plants. The Plant Journal, **45**(4), 651–683 (2006).

S6. Patel M. K., Das S. & Thakur J. K. GC-MS-based analysis of methanol:chloroform-extracted fatty acids from plant tissues. *Bio-protocol* 8, e3014 (2018).

S7. Czechowski T., Stitt M., Altmann T., Udvardi M. K. & Scheible W. R. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiol.* **139**, 5–17 (2005).

S8. Langmead B., Trapnell C., Pop M. & Salzberg S. L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* **10**, R25 (2009).

S9. Robinson M. D., McCarthy D. J. & Smyth G. K. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140 (2010).

S10. Zhou Y., Zhou B., Pache L., Chang M., Khodabakhshi A. H., Tanaseichuk O. et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat. Commun.* **10**, 1523 (2019)..

S11. Wang H., Li S., Li Y., Xu Y., Wang Y., Zhang R. et al. MED25 connects enhancer-promoter looping and MYC2-dependent activation of jasmonate signalling. *Nat. Plants* **5**(6), 616–625 (2019).

S12. Langmead B. & Salzberg S. L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9**, 357–359 (2012).

S13. O'Malley R. C., Huang S. C., Song L., Lewsey M. G., Bartlett A., Nery J. R. et al. Cistrome and epicistrome features shape the regulatory DNA landscape. *Cell* **165**(5), 1280–1292 (2016).

S14. Zhang Y., Liu T., Meyer C. A., Eeckhoute J., Johnson D. S., Bernstein B. E. et al. Model-based Analysis of ChIP-Seq (MACS). *Genome Biol.* 9, R137 (2008).

Supplementary Figures

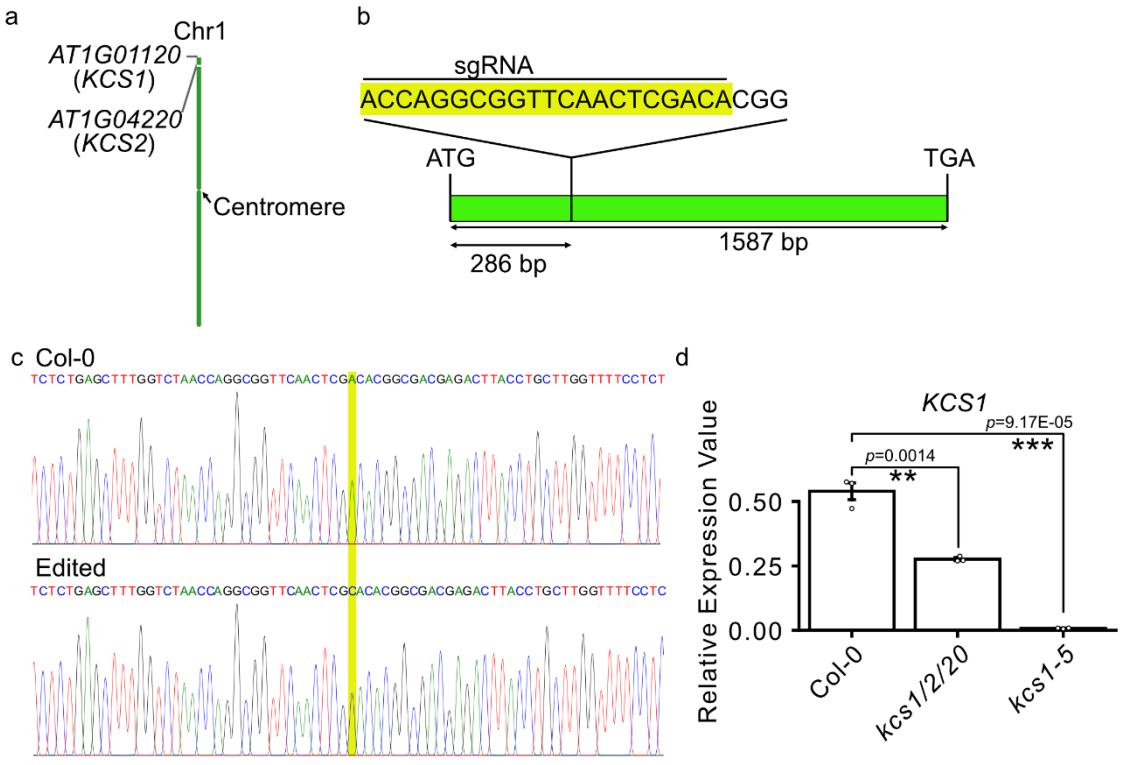


Fig. S1 Information of *KCS1* genome edited line. **A.** *KCS1* and *KCS2* genome location in *A. thaliana*. **B.** Genomic position of the *KCS1* gene targeted by the designed guide RNA. The sgRNA sequence highlighted in yellow is located 286 bp downstream of the start codon (ATG), and KCS1 has a full-length coding region of 1,587 bp. **C.** Sequencing results of the *kcs1* genome edited line. The site where a single "C" was inserted by genome editing was highlighted in yellow. **D.** Expression level of *KCS1* in Col-0, *kcs* genome edited line (*kcs1/2/20*), and *kcs1-5*. Significant differences from the roots of Col-0 plants grown at 22 °C, determined using the Student's *t*-test (**$p < 0.01$, ***$p < 0.001$).

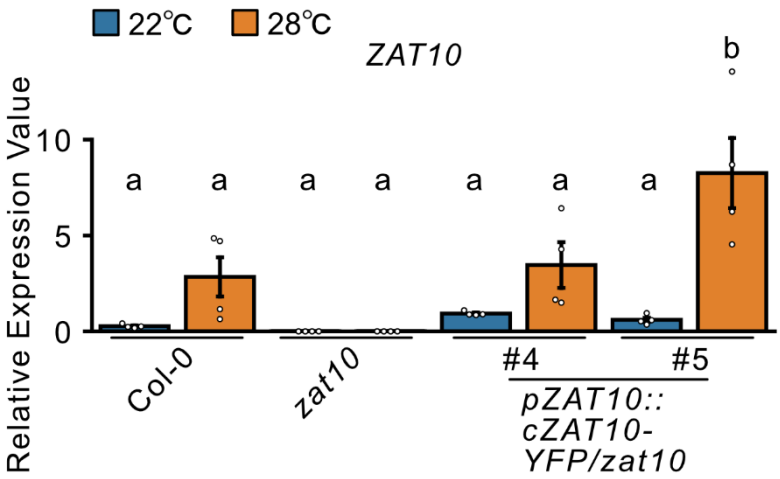
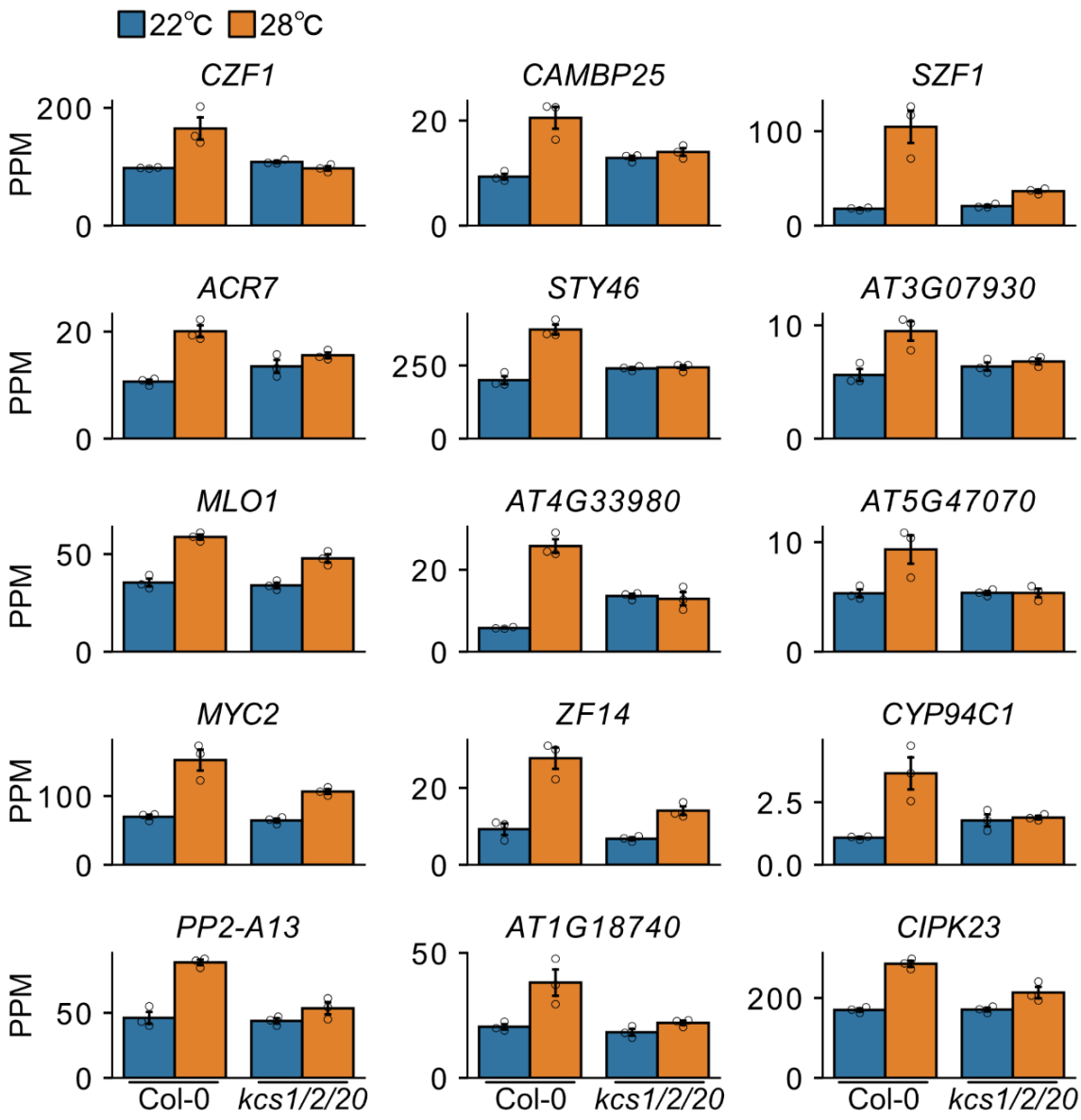
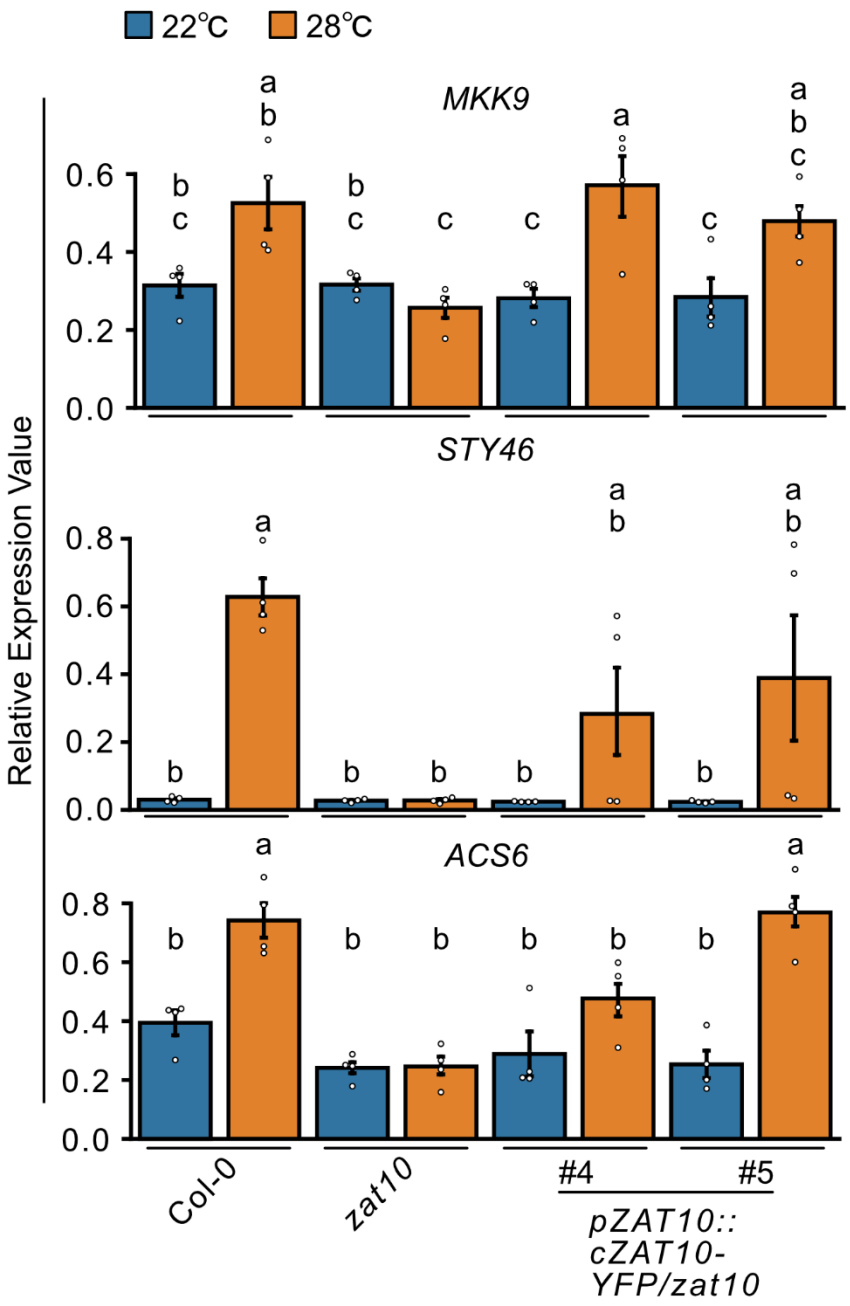


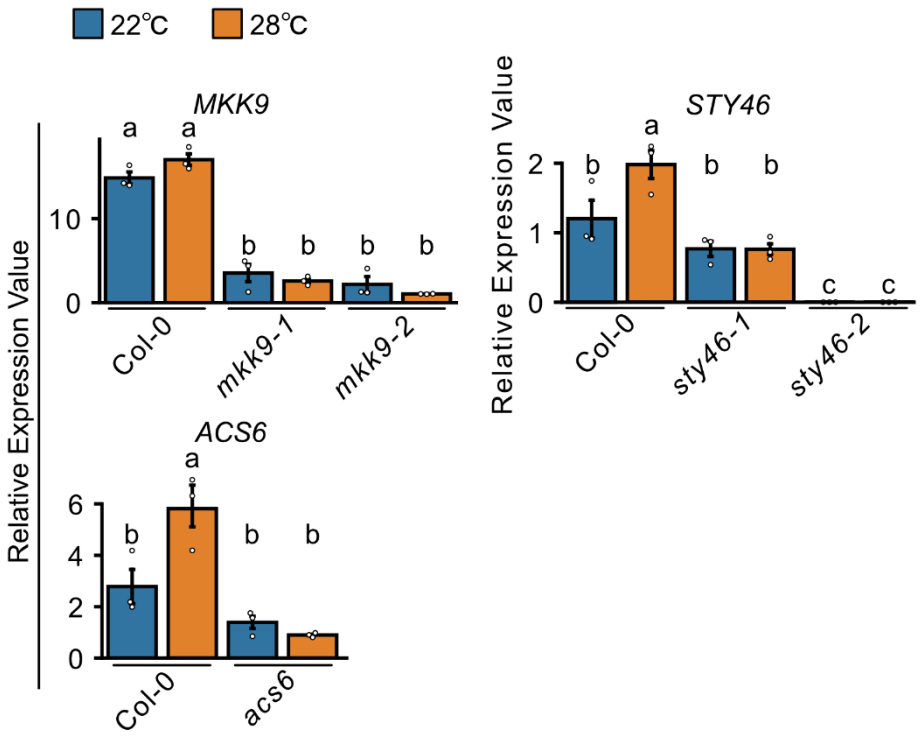
Fig. S2 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to detect *ZAT10* in the roots of 10-days-old seedling of Col-0, *zat10*, and two independent *ZAT10* complementation lines grown at 22 and 28 °C (n = 4, ± standard deviation [SD]). Letters above the boxes indicate statistically significant differences between samples, determined using the Tukey's honestly significant difference (HSD) test ($p < 0.05$).



169 **Fig. S3** Expression levels of *ZAT10* target genes associated stress response in Col-0 and *kcs1/2/20*
170 grown at 22 and 28 °C. Data was retrieved from our RNA-seq data set.



171 **Fig. S4** Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to detect the
172 expression of MYC2 target genes in the roots of 10-days-old seedling of Col-0, *zat10*, and ZAT10
173 complementation lines grown at 22 and 28 °C (n = 4, ± standard deviation [SD]). Letters above the
174 boxes indicate statistically significant differences between samples, determined using the Tukey's
175 honestly significant difference (HSD) test ($p < 0.05$).



176 **Fig. S5** *MKK9*, *ACS6*, and *STY46* expression in each mutant. Reverse transcription-quantitative
177 polymerase chain reaction (RT-qPCR) to detect *MKK9*, *ACS6*, and *STY46* expression in the roots in
178 10-days-old seedlings of Col-0, *mkk9-1, mkk9-2*, *sty46-1*, *sty46-2*, and *acs6* grown at 22 and 28 °C (n
179 = 3, ± standard deviation [SD]). Letters above the boxes indicate statistically significant differences
180 between samples, determined using the Tukey's honestly significant difference (HSD) test ($p < 0.05$).
181
182
183 **Table S1.** Primers used in this study.
184 **Genotyping Primer**

Primer name	Sequence(5' to 3')	purpose
SALK LB	TTTCGCCTGCTGGGGCAAACCAG	SALK T-DNA
SAIL LB	GCTTCCTATTATATCTTCCCAAATTAC CAATACA	Sail T-DNA
S200839(kcs1-5)-LP	TGCTCTGACAATGGAAGAACC	S200839(kcs1-5)
S200839(kcs1-5)-RP	TTCATCATCGGCCGTTATAAG	S200839(kcs1-5)
CS71727(KCS2)_Fw	GGAACCTTCGAGGATGACTTTGAACC	CS71727(KCS2)
CS71727(KCS2)_Rv	CCCCTTCGAGATTCCGTTATCTTTTG	CS71727(KCS2)
CS71728(KCS20)_Fw	CGCGGTTCTTGAGAAGACCGGTGTGA	CS71728(KCS20)
CS71728(KCS20)_Rv	TAAAGACTACAAAGCCTGTCACTGTC	CS71728(KCS20)
SALK_054092(zat10)-LP	TATTTTGTAAGGCGGCATCAG	SALK_054092(zat10)

SALK_054092(zat10)-RP	AAGTCAAACCGAGGCTTCTTC	SALK_054092(zat10)
SALK017005(myc2)-LP	GGCGGGATTTAATCAAGAGAC	SALK017005(myc2)
SALK017005(myc2)-RP	TTTGGTACAACCGCTCGTAAC	SALK017005(myc2)
CS16569(acs6)_LP	CACTTGGTGAACAATCACACG	CS16569(acs6)
CS16569(acs6)_RP	GCTTGCCTGAATTCAGACAAG	CS16569(acs6)
SALK_112195(sty46-1)_LP	TTGGCTATCTCTTGAAGCTGC	SALK_112195(sty46-1)
SALK_112195(sty46-1)_RP	AAGCAAAAGGGCGTCTTTAAG	SALK_112195(sty46-1)
CS887714(sty46-2)_LP	GTCTGCAACCTTAACCACCTG	CS887714(sty46-2)
CS887714(sty46-2)_RP	CAATCCTTCTCTCCGGAAAAG	CS887714(sty46-2)
CS872513(mkk9-1)_LP	GGCTTTAGTACGTGAACGTCG	CS872513(mkk9-1)
CS872513(mkk9-1)_RP	CCCAAAACTTATGTACACGATTG	CS872513(mkk9-1)
SALK_017378(mkk9-2)_LP	CGATGAACGATCTTAAGAGCG	SALK_017378(mkk9-2)
SALK_017378(mkk9-2)_RP	TTAGAAGCTTTTCCACGTTGG	SALK_017378(mkk9-2)

185

Cloning Primers

Primer name	Sequence (5' to 3')	purpose
pZAT10_HiFi_Fw	GTTGGCTCCGAATTCGCCTTCATGCACACACAGAGGAGAGAGTT	Cloningfor*ZAT10* promoter region
pZAT10_HiFi_Rv	TTGGGTCGAATTCGCCCTTTTAAGTTAAAGATTCTGAGGATTTCTTGTCTCTCG	Cloning for *ZAT10* promoter region
gZAT10_HiFi_Fw	TGTACAAAAAAGCAGGCTTTATGGCGCTCGAGGCTCTTAC	Cloning for *ZAT10* in pDonr201-YFP
gZAT10_HiFi_Rv	TCCTCGCCCTTGCTCACCATAAGTTGAAGTTTGACCGGAAAGTCAAAC	Cloning for *ZAT10* in pDonr201-YFP
pKCS1_HiFi_Fw	TCCGAATTCGCCCTTTATTATGCTGAAAGGTCTAG	Cloning for *KCS1* promoter region
pKCS1_HiFi_Rv	GTCGAATTCGCCCTTTTTGGTTGAACCGGATAATCA	Cloning for *KCS1* promoter region
pKCS2_HiFi_Fw	TCCGAATTCGCCCTTTAAAACGCTGCGTTTCTCAC	Cloning for *KCS2* promoter region
pKCS2_HiFi_Rv	GTCGAATTCGCCCTTTGGTAGGTTTTTTGGTTTTAA	Cloning for *KCS2* promoter region

cKCS1_HiFi_Fw	TGTACAAAAAAGCAGGCTTTATGGAGA GAACAAACAGCATTGAGA	Cloning for *KCS1* in pDonr201-YFP
cKCS1_HiFi_Rv	TCCTCGCCCTTGCTCACCATTTGCACAAC TTTAACCGGATATTGATCA	Cloning for *KCS1* in pDonr201-YFP
cKCS2_HiFi_Fw	TGTACAAAAAAGCAGGCTTTATGAATGA GAATCACATTCAAAGTGATCACA	Cloning for *KCS2* in pDonr201-YFP
cKCS2_HiFi_Rv	TCCTCGCCCTTGCTCACCATTCGAGATTC CGAGGAAGATGTCAC	Cloning for *KCS2* in pDonr201-YFP
pENTR-5-Fw	AAGGGCGAATTCGGAGCCAACTTT	Promoter replacement for recombination
pENTR-5-Rv	AAGGGCGAATTCGACCCAAGTTTGTACA	Promoter replacement for recombination
pDonr201-YFP-Fw	ATGGTGAGCAAGGGCGAGGA	Cloning for *pDonr201-YFP*
pDonr201-YFP-Rv	AAAGCCTGCTTTTTTGTACAAACTTGG	Cloning for *pDonr201-YFP*

186

qPCR Primers

Primer name	Sequence (5' to 3')	purpose
qPDF2 Fw	TAACGTGGCCAAAATGATGC	qPCR for *PDF2*
qPDF2 Rv	GTTCTCCACAACCGCTTGGT	qPCR for *PDF2*
qKCS1_Fw	AGATTGCGTTTGGATCGGGT	qPCR for *KCS1*
qKCS1_Rv	TTACCGGTCATCTCCTCCGT	qPCR for *KCS1*
qKCS2_Fw	CCATTGATCTCGCTAAACAGC	qPCR for *KCS2*
qKCS2_Rv	TCGGTCGTTGCCTAAATACC	qPCR for *KCS2*
qKCS20_Fw	CCAACACCGTCACTTTCTGC	qPCR for *KCS20*
qKCS20_Rv	GGCCAGCACTACATCCCATT	qPCR for *KCS20*
qZAT10_Fw	TCACAAGGCAAGCCACCGTAAG	qPCR for *ZAT10*
qZAT10_Rv	TTGTCGCCGACGAGGTTGAATG	qPCR for *ZAT10*
qMYC2_Fw	CAGGAGGAGTGTTTGGGATGC	qPCR for *MYC2*
qMYC2_Rv	GTCGAAAAATTAAGTTCTCGGGAG	qPCR for *MYC2*
qACS6_Fw	AGGGTGGTTCAGATCGCAAG	qPCR for *ACS6*
qACS6_Rv	GCTCTCGCGGATAAACTCGT	qPCR for *ACS6*
qMKK9_Fw	GTGTGTTTTGGAGAACCGCC	qPCR for *ACS6*
qMKK9_Rv	CCGTCCATCGCTTACTCGAA	qPCR for *ACS6*

qSTY46_Fw	CGCATCCGAAATTGGCAGAG	qPCR for *STY46*
qSTY46_Rv	TCTTCTCCTACCTCCTCCTTGGCT	qPCR for *STY46*

Dataset S1. Significant differentially expressed genes between Col-0 and *ksc1/2/20* grown at 22 and 28 °C.

Dataset S2. List of genes bound by ZAT10 within 1,000 bp upstream of the translation start site.

Dataset S3. Gene ontology (GO) terms of genes overlapping between the ZAT10 cistrome and the set of genes specifically upregulated at 28 °C in Col-0, based on our RNA-seq data.

Dataset S4. List of genes bound by MYC2 within 1,000 bp upstream of the translation start site.

Dataset S5. List of genes overlapping between the MYC2 ChIP-seq and the set of genes specifically upregulated at 28 °C in Col-0, based on our RNA-seq data.