

MOS4-associated complex contributes to proper splicing and suppression of ER stress under long-term heat stress in Arabidopsis

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Abstract

Plants are often exposed not only to short-term (S-) but also to long-term (L-) heat stress over several consecutive days. A few Arabidopsis mutants defective in L-heat tolerance have been identified, but the molecular mechanisms are less understood for this tolerance than for S-heat stress tolerance. To elucidate the mechanisms of the former, we used a forward genetic screen for *sensitive to long-term heat* (*sloh*) mutants and isolated *sloh3* and *sloh63*. The mutants were hypersensitive to L- but not to S-heat stress, and *sloh63* was also hypersensitive to salt stress. We identified the causal genes, *SLOH3* and *SLOH63*, both of which encoded splicing-related components of the MOS4-associated complex (MAC). This complex is widely conserved in eukaryotes and has been suggested to interact with spliceosomes. Both genes were induced by L-heat stress in a time-dependent manner, and some abnormal splicing events were observed in both mutants under L-heat stress. In addition, endoplasmic reticulum (ER) stress and subsequent unfolded protein response occurred in both mutants under L-heat stress and were especially prominent in *sloh63*, suggesting that enhanced ER stress is due to the salt hypersensitivity of *sloh63*. Splicing inhibitor pladienolide B led to concentration-dependent disturbance of splicing, decreased L-heat tolerance, and enhanced ER stress. These findings suggest that maintenance of precise mRNA splicing under L-heat stress by the MAC is important for L-heat tolerance and suppressing ER stress in Arabidopsis.

Keywords: heat tolerance, long-term heat stress, selective splicing, ER stress, Arabidopsis

Significance Statement

Plants are exposed not only to short-term (S-) but also to long-term (L-) heat stress, however, the L-heat stress response is not yet well understood. Using forward genetic screening, we isolated *sloh3* and *sloh63* mutants, which are hypersensitive to L-heat but not to S-heat stress. *SLOH3* and *SLOH63* were identical to the splicing-related factor genes *MAC9* and *MAC17*, respectively. Splicing inhibitor pladienolide B led to concentration-dependent disturbance of splicing, decreased L-heat tolerance, and enhanced ER stress. These findings suggest that maintenance of appropriate mRNA splicing and suppression of ER stress under L-heat stress are important for L-heat tolerance in Arabidopsis.

Introduction

High temperature is a serious stress that affects crop growth and production. The yield of major cereal crops has reportedly decreased due to recent temperature increases [1], so it is important to elucidate the mechanisms of plant responses to heat stress and to generate heat-tolerant crops. In *Arabidopsis thaliana*, a dynamic transcriptional network is induced within 1 h through proteins of the heat shock factor A1 (HsfA1) group. Under short-term (S-) heat stress (42°C for 30–50 min), this group regulates most heat stress-responsive genes including those for heat shock proteins (HSPs).

In Arabidopsis, the HsfA1 group consists of *HsfA1a*, *-b*, *-d*, and *-e*. The *hsfa1a/b/d* triple mutation impairs the global induction of heat-responsive genes and reduces heat tolerance [2–4]. Conversely, *HsfA1* overexpression induces the expression of various heat-responsive genes and improves S-heat tolerance [5]. Plants exhibit S-heat tolerance after heat acclimation, in which exposure to moderately high temperatures enables plants to acquire tolerance to subsequent otherwise lethal high temperatures [3]. In Arabidopsis, HsfA2 is necessary for the maintenance of acquired thermotolerance. The transient binding of HsfA2 to HSP

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genes results in a sustained increase in histone marks (such as histone H3 lysine 4 trimethylation; H3K4me3), which promotes HSP gene expression [6, 7]. After heat exposure, HsfA2 levels gradually decrease, while H3K4me3 levels and HSP expression remain high. JMJ protein-mediated sustained H3K27me3 demethylation on small chaperone-encoding HSP genes that function as memory genes controls heat memory [8].

Plants are also exposed to long-term (L-) heat stress, which can involve temperatures up to 37°C over a period of several days. While the molecular mechanisms for responding to S-heat stress are well-characterized as described above, the regulation of L-heat stress tolerance is not fully understood. To investigate the contribution of HSPs and Hsfs to L-heat tolerance, we previously evaluated the L-heat tolerance of the *hsp90*, *hsp101*, *hsfa1d*, *hsfa1e*, and *hsfa2* mutants (Isono et al. [9]). Only *hsfa1d* was slightly more sensitive to L-heat stress than the WT, whereas the other mutants were comparable to WT, implying that different genes and mechanisms contribute to S-heat and L-heat tolerances.

A forward genetic approach identified a few *Arabidopsis* mutants including *heat intolerant* (*hit*) mutants hypersensitive to L-heat stress [10–13]. To better understand the mechanism for L-heat tolerance, we have also isolated a *sensitive to long-term heat* (*sloh*) mutant from EMS-mutagenized seeds of Col-0, an L-heat-tolerant *A. thaliana* accession [9, 14]. The causal gene of *sloh4* is identical to *MIP3*, which encodes a member of the

MAIGO2 (MAG2) tethering complex localized at the endoplasmic reticulum (ER) membrane; that of *hit1-1* encodes a protein identical to the homolog of yeast Vsp53p, a member of the GARP tethering complex localized at the Golgi [9, 10, 12]. Both mutants are hypersensitive to L-heat and salt stresses but not to S-heat stress [9, 12]. Under L-heat stress, ER stress and the following unfolded protein response (UPR) are more pronounced in *sloh4* than in wild type (WT) [9].

Alternative splicing occurs in all eukaryotes and enhances transcriptional plasticity and proteome diversity in response to various growth and stress cues including heat stress [15]. Alternative splicing is classified into five types: exon skipping, mutually exclusive exons, alternative 5' splice site, alternative 3' splice site, and intron retention [15]. Exon skipping is the major type in mammals, whereas intron retention is the major type in plants [16]. Intron retention is thought to enrich proteome diversity in mammals, whereas it produces nonfunctional isoforms in plants [17–19]. The spliceosome is composed of five core subcomplexes: the U1, U2, U4, U5, and U6 small ribonucleoproteins (snRNPs) [20]. Each U snRNP forms a ring structure composed of a corresponding snRNA and an SM-like (LSM) family protein [21]. The activation and catalysis of splicing are largely mediated by the NineTeen complex in humans and yeast or MOS4-associated complex (MAC) in *Arabidopsis* [22, 23]. The MAC is necessary to define the specificity of the interaction of U5 and U6 with the 5' splice site [24]. The knockout mutant of *LSM5* is hypersensitive to abscisic acid (ABA), NaCl, and S-heat stress (42°C for 90 min) [25, 26]. The mutant of a U5-snRNP-interacting protein homolog, *STABILIZED1* (*STA1*), is hypersensitive to heat (38°C for 1 day) [27]. In both mutants, premature RNA levels of *heat shock factor A3* (*HsfA3*) are increased, while its mature RNA levels are decreased. However, the overexpression of *HsfA3* in the *sta1-1* background is unable to complement the heat-sensitive phenotype of this mutant, suggesting that *STA1* affects broad transcriptional regulation other than that of the *HsfA3* regulon [27]. Thus, knowledge of the regulatory mechanism underlying premature mRNA splicing under environmental stress conditions in plants is still fragmentary.

In this study, to elucidate the mechanisms of L-heat stress responses, we used a forward genetic screen for *sloh* mutants and isolated *sloh3* and *sloh63*. The causal genes both encoded splicing-related factors identified as components of the MOS4-associated complex (MAC), which is widely conserved in eukaryotes and has been suggested to interact with spliceosomes. Under L-heat stress, *sloh3* and *sloh63* exhibited some abnormal splicing events and enhanced ER stress responses. Our findings suggest that maintenance of precise mRNA splicing under L-heat stress is important for L-heat tolerance and suppressing ER stress in *Arabidopsis*.

Results

Isolation and characterization of *sloh3* and *sloh63* mutants

When 10-day-old seedlings were exposed to 37°C for 3 days, *A. thaliana* Col-0 WT did not show significant growth inhibition (Fig. 1A). We generated an EMS-mutagenized Col-0 population and screened 3,000 M2 seedlings for *sloh* mutants, which exhibits growth inhibition for 3 days at 37°C. We isolated *sloh3* and *sloh63* as L-heat-sensitive mutants by the forward genetic screening (Fig. 1A). The sensitivity to L-heat stress was slightly and not

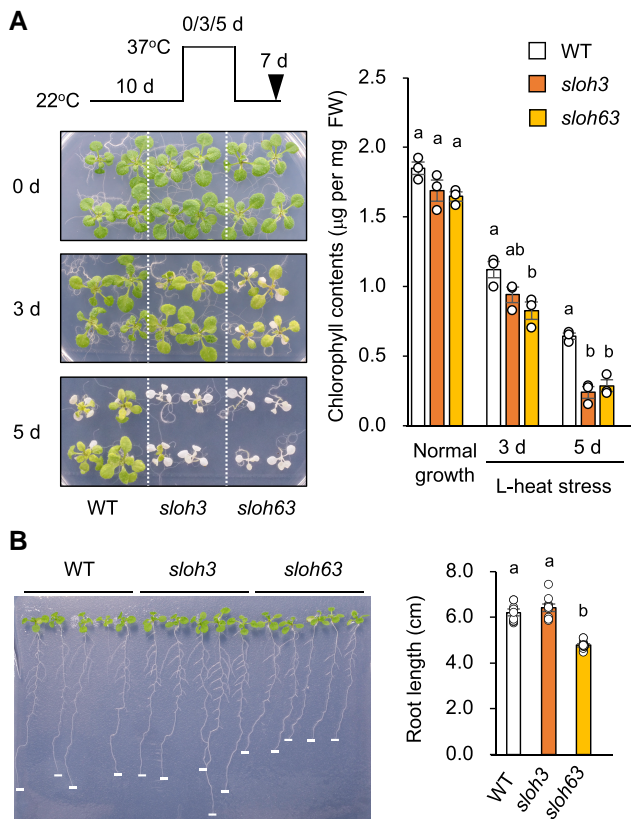


Fig. 1. Identification of the *sloh3* and *sloh63* mutants. A) L-heat tolerance of the mutants. Ten-day-old wild-type (WT) and mutant seedlings grown at 22°C were placed at 37°C for 0, 3, or 5 days, as indicated, and then returned to 22°C for another 7 days. Left: plant images at the time point indicated with an arrowhead. Right: chlorophyll contents. B) Root length. Seedlings were grown vertically at 22°C for 14 days. Data are mean \pm SE, $n = 3$. The same letters above columns indicate no significant difference within a time point in one-way ANOVA with post hoc Tukey HSD test, $P < 0.05$.

significantly higher in *sloh63* than in *sloh3* (Fig. 1A). Roots were shorter in *sloh63* than in WT or *sloh3* (Fig. 1B).

Both mutants did not differ significantly from WT in their tolerance to S-heat stress or to S-heat stress after heat acclimation (Figs. 2A, S1) or in the expression profiles of *HsfA1d* (except in

sloh3 at 24 h), *HsfA2* (except in *sloh63* at 1 h), and *HSP70* under heat stress (Fig. 2B). Sensitivity to NaCl and LiCl stress (Li^+ is more toxic than Na^+) was higher in *sloh63* than in WT and *sloh3*, but osmo-tolerance did not differ significantly among them (Fig. 2C). These results suggest that *sloh3* is specifically defective in L-heat stress response, and that *sloh63* is defective in L-heat stress response, salt stress response, and root elongation.

Identification of the causal genes of *sloh3* and *sloh63*

To identify the causal genes of *sloh3* and *sloh63*, each mutant was crossed with Da (1)-12, an Arabidopsis accession moderately tolerant to L-heat. Using F_2 progeny, we mapped the causal loci near the simple-sequence-length polymorphism (SSLP) markers at 10,608 kb in the middle of chromosome 5 for *sloh3* and at 18,084 kb close to the distal end of the long arm of chromosome 2 for *sloh63* (Fig. 3A). To detect all mutations within the mapped regions, we sequenced the *sloh3* and *sloh63* genomes and found only one nonsynonymous mutation in the coding region of each mutant: a mutation in the second exon of *At5g28740* led to a D284N substitution in *sloh3*, and a nonsense mutation in the exon of *At2g43770* led to W299X (X, stop codon) and a truncated protein in *sloh63* (Fig. 3A). To perform a complementation test, we transformed *sloh3* and *sloh63* with *At5g22350* and *At2g43770*, respectively, including its native promoter region. In each complementation line, the expression level was about 2-fold higher than that of the WT, which is reasonable as the sum of the expression of the native gene-derived expression and that of the transgene (Fig. 3B). The introduction of *At5g28740* into *sloh3* and *At2g43770* into *sloh63* restored the L-heat tolerance to that of WT, indicating that these genes were the causal genes of interest (Fig. 3B). To understand whether *SLOH3* and *SLOH63* are involved in the L-heat stress response, we examined their expression profiles under L-heat stress. The levels of both transcripts were increased by L-heat stress in a time-dependent manner (Fig. 3C). In our previous studies, defects in ER-Golgi transport and very-long-chain fatty acid synthesis impaired not only L-heat tolerance but also other abiotic stress tolerances such as salt and osmotic stress [9, 28]. This suggests that the mechanism for L-heat tolerance in Arabidopsis is related to other stress tolerance mechanisms. To determine whether the *sloh3* and *sloh63* phenotypes are specific to L-heat tolerance, we evaluated tolerance to osmotic (sorbitol) and salinity (NaCl and LiCl) stresses. *SLOH63* also complemented the NaCl and LiCl hypersensitivity and shorter roots of *sloh63*, suggesting that the mutation in this gene is responsible for these phenotypes (Figs. 3D, S2, S3).

Effects of *SLOH3* and *SLOH63* on transcriptional regulation

SLOH3 and *SLOH63* were identical to *MOS4-ASSOCIATED COMPLEX* 9 and 17 (*MAC9* and *MAC17*), respectively, which encode an RNA-binding proteins with an RNA helicase domain and an intron-binding domain. MAC proteins are widely conserved in eukaryotes and interact with spliceosomes [22, 29, 30]. *MAC9* and *MAC17* regulate the expression levels of plant disease response genes and are involved in miRNA biosynthesis and mRNA splicing, but their involvement in responses to heat and other abiotic stresses is unknown. As both genes encode RNA-binding proteins, the loss of their function may affect alternative splicing under L-heat stress. To test this hypothesis, we performed qRT-PCR of the *BAG6*, *bZIP28*, and *MEMB11* genes, which retain their introns

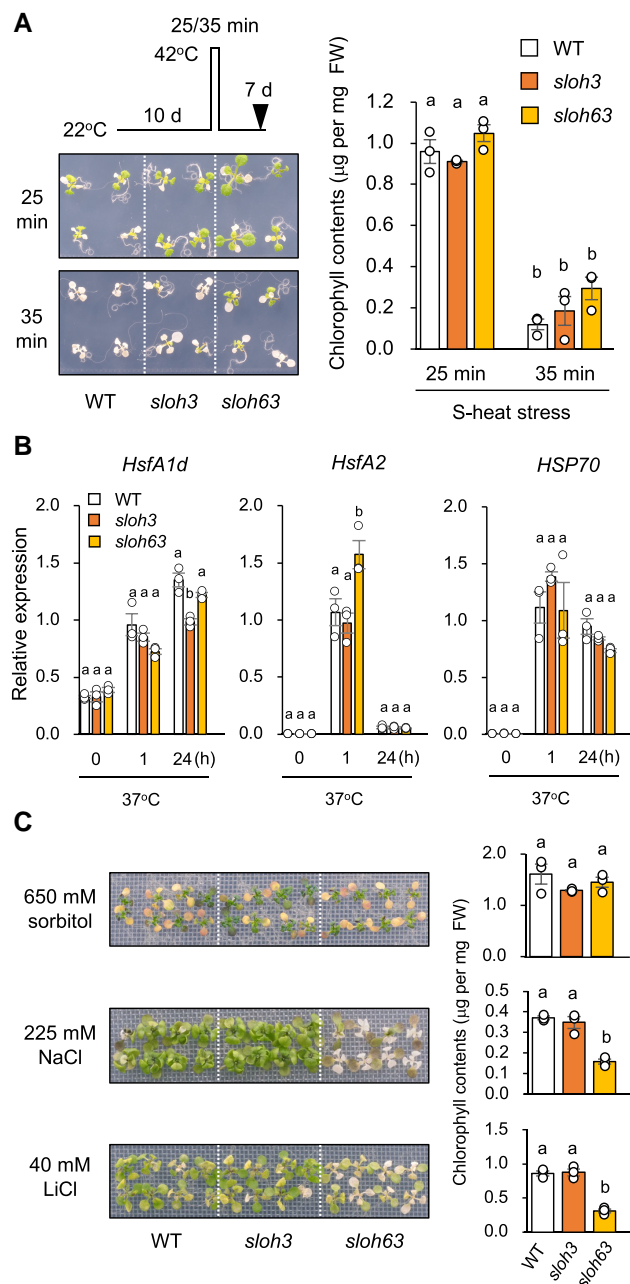


Fig. 2. Characterization of the *sloh3* and *sloh63* mutants. A) S-heat tolerance. Ten-day-old wild-type (WT) and mutant seedlings grown at 22°C (normal conditions) were placed at 42°C for 25 or 35 min and returned to 22°C for 7 days. Left: plant images. Right: chlorophyll contents (mean ± SE, n = 3). B) Expression of *HsfA1d*, *HsfA2*, and *HSP70* under normal and heat-stress conditions; expression relative to *ACTIN2* was determined by qRT-PCR. Ten-day-old seedlings grown at 22°C (0 h) were placed at 37°C for 1 or 24 h. C) Osmotic, NaCl, and LiCl stress tolerance. Ten-day-old seedlings grown at 22°C were mesh-transferred onto MS agar plates containing 650 mM sorbitol for 21 days (osmotic tolerance), 225 mM NaCl for 11 days (NaCl tolerance), or 40 mM LiCl for 8 days (LiCl tolerance). Left: plant images. Right: chlorophyll content. Data are mean ± SE, n = 3. The same letters above columns indicate no significant difference within a time point in one-way ANOVA with post hoc Tukey HSD test, P < 0.05.

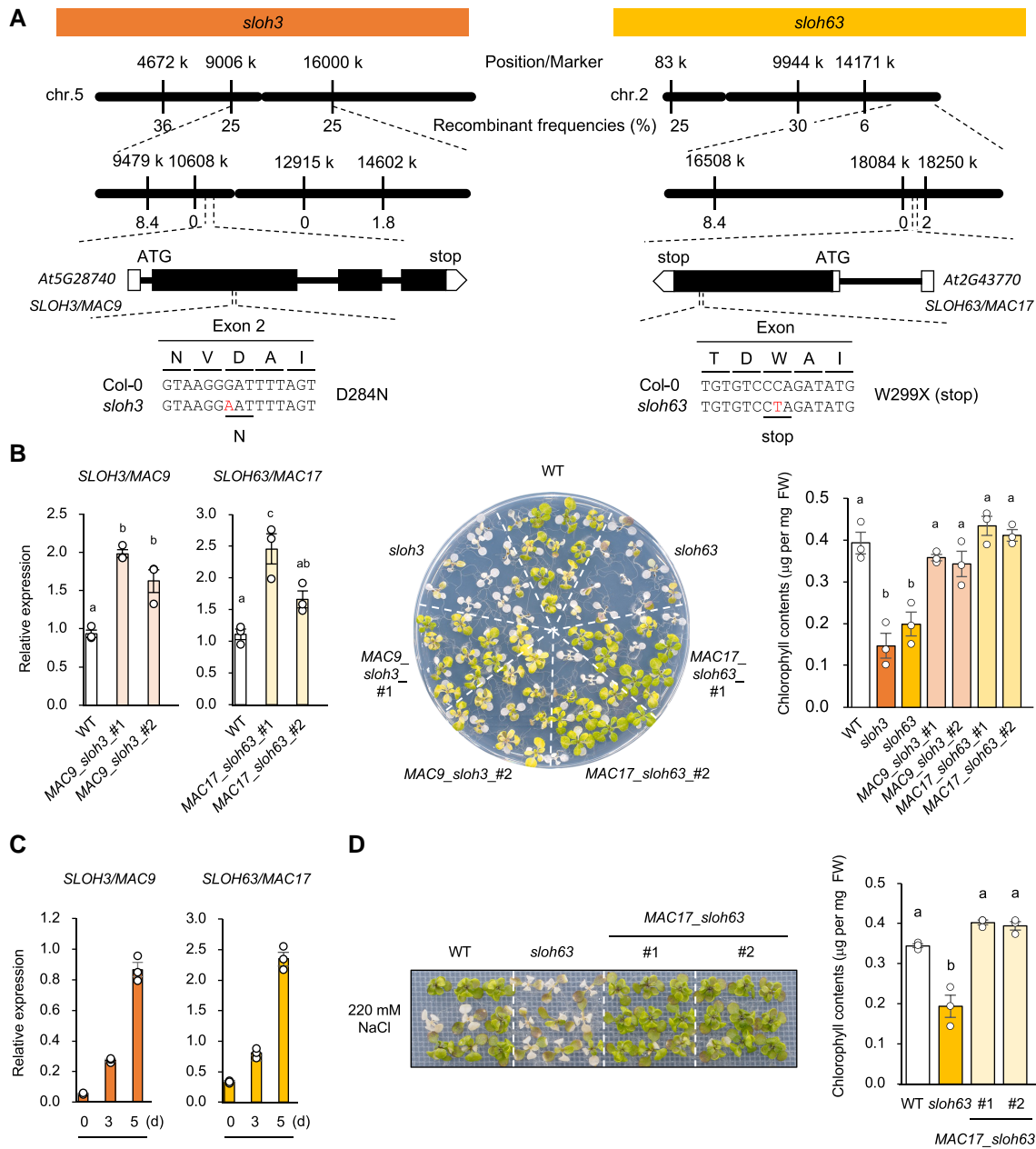


Fig. 3. Identification of the causal genes of *sloh3* and *sloh63*. **A**) High-resolution mapping of the causal loci. Mutations are shown in red. **B**) L-heat tolerance of wild-type (WT), *sloh3*, *sloh63*, *sloh3* lines complemented by transformation with *MAC9* (*MAC9_sloh3*_#1, 2), and *sloh63* lines complemented by transformation with *MAC17* (*MAC17_sloh63*_#1, 2). Left: Relative expression of *SLOH3/MAC9/At5g22350* or *SLOH63/MAC17/At2g43770* in the complementation lines under normal conditions; expression levels were determined by quantitative real-time PCR relative to those of *Actin2* (mean \pm SE, $n = 3$). Middle: plant images. Right: Chlorophyll contents of WT, mutants, and complemented lines. **C**) Expression of *SLOH3/MAC9* and *SLOH63/MAC17* in WT under L-heat stress. *ACTIN2* was used as an internal standard. **D**) NaCl tolerance of WT, *sloh63*, and *sloh63* lines complemented by transformation with *MAC17* (*MAC17_sloh63*_#1, 2). Left: plant images. Right: chlorophyll contents. **B–D**) Data are mean \pm SE, $n = 3$. The same letters above columns indicate no significant difference within a time point in one-way ANOVA with post hoc Tukey HSD test, $P < 0.05$.

under heat stress [28]. All three genes had higher premature mRNA levels in *sloh3* and *sloh63* than in WT under L-heat stress (Fig. 4); *sloh3* showed the earliest increase in premature mRNA level under L-heat stress.

The mature mRNA level of *bZIP28*, a key activator of the ER stress response [31], was higher in *sloh63* than in WT and *sloh3* under L-heat stress (Fig. 4). Defects in ER-to-Golgi transport and biosynthesis of very-long-chain fatty acids, which enhance ER stress, impair not only L-heat tolerance but also salt tolerance in Arabidopsis [9, 28]. To examine differences in the ER stress

response among WT and mutants under normal and L-heat stress conditions, we examined the transcript levels of *bZIP28*-regulated genes (*CRT2*, *P58IPK*, and *SDF2*) and other key activators of the ER stress response (*bZIP60* and *bZIP60*-regulated genes *PDI9* and *SAR1A*) [32]. The transcript levels of these genes were highest at day 3 of heat stress in *sloh3* and at day 5 in *sloh63* (Fig. 5). *bZIP60* induces the UPR triggered by splicing of *bZIP60* [33, 34]. The transcript level of the spliced (active) *bZIP60* form detected by RT-PCR was only slightly higher in *sloh3* and *sloh63* than in WT under L-heat stress (Fig. S2).

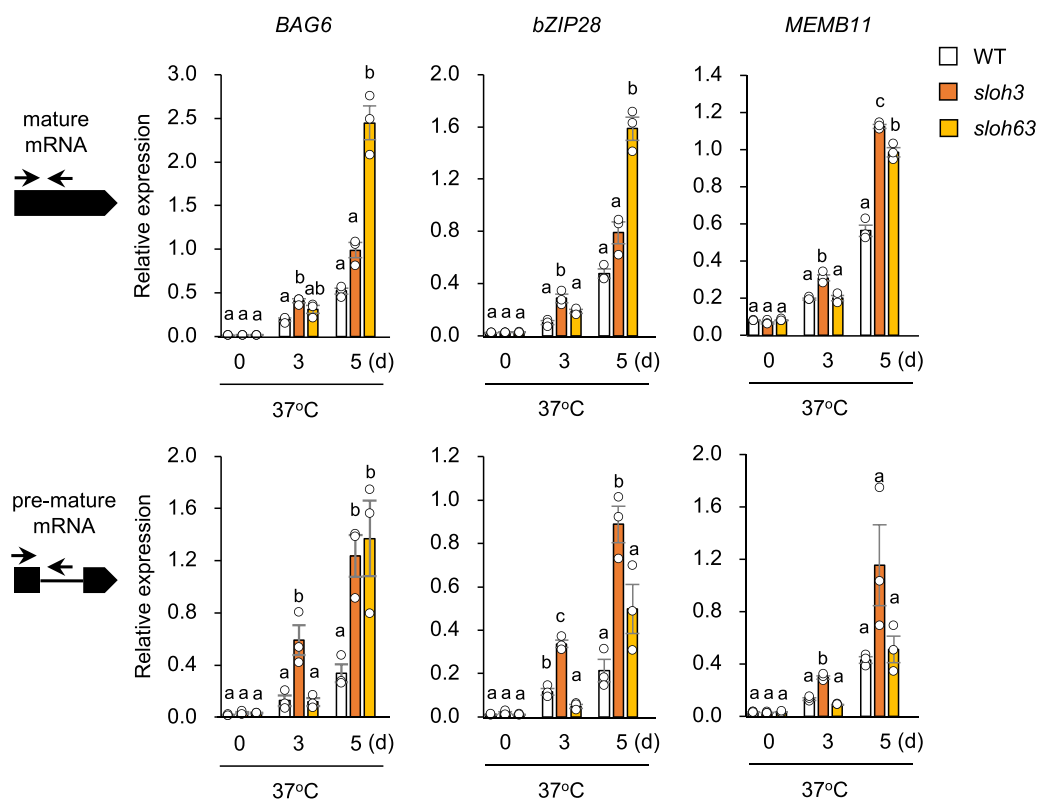


Fig. 4. Detection of retained introns in heat-responsive genes under L-heat stress in *sloh3* and *sloh63*. Expression of mature mRNAs (upper panels) and premature mRNAs (lower panels) of *BAG6*, *bZIP28*, and *MEMB11* was measured in wild type (WT), *sloh3*, and *sloh63* Ten-day-old seedlings grown at 22°C (0 day) were placed at 37°C for 3 or 5 days. Data are mean \pm SE, $n = 3$. The same letters above columns indicate no significant difference within a time point in one-way ANOVA with Tukey test, $P < 0.05$.

Effects of a splicing inhibitor on L-heat stress tolerance and ER stress response

To determine whether disturbing splicing machinery impairs L-heat tolerance and whether an increase in premature mRNAs induces ER stress, we evaluated the effects of a splicing inhibitor on L-heat tolerance and ER stress. Pladienolide B (PB) targets the splicing factor SF3b in humans, an ortholog of MAC18 in Arabidopsis, and inhibits constitutive and alternative splicing [35, 36]. The levels of premature mRNAs of *BAG6*, *bZIP28*, and *MEMB11* and mature mRNA of *BAG6* and *bZIP28* were increased by PB in a concentration-dependent manner (Fig. 6A). We treated WT plants with PB for 1 day and subjected them to L-heat stress for 5 days and found that L-heat tolerance was impaired by PB (Fig. 6B). To determine whether ER stress is induced by splicing inhibition, we analyzed the expression levels of ER stress response genes in PB-treated WT plants. The transcript levels of all six genes tested were upregulated by PB; *bZIP60* and *PDI9* were upregulated in a PB concentration-dependent manner (Fig. 6C). These findings suggest that inhibition of splicing induces ER stress.

Effects of L-heat stress on gene expression in *sloh3* and *sloh63*

To investigate the mechanisms underlying the heat sensitive phenotype of *sloh3* and *sloh63*, the RNAseq analysis was performed on WT, *sloh3* and *sloh63* seedlings under normal or heat stress (37°C 3 day) conditions. The differentially expressed genes (DEGs) between normal and heat stress conditions in WT, *sloh3* and *sloh63* were identified and classified them as shown in the Venn diagram

in Fig. 7 (Fig. 7A, Table S1). To characterize DEGs in *sloh3* and *sloh63*, a GENEONTOLOGY (GO) enrichment analysis was performed for *sloh3*-specific, *sloh63*-specific, or *sloh3* and *sloh63* common DEGs (Fig. 7B, C). As a result, no significant GO terms were detected for *sloh3*-specific DEGs, but significant GO terms were successfully obtained for *sloh63*-specific or *sloh3* and *sloh63* common DEGs. GO terms related to protein, peptide, and amide metabolism were enriched for the *sloh3* and *sloh63* common DEGs (Fig. 7B). It is known that abnormal splicing results in ER stress due to the accumulation of abnormal proteins, which in turn results in their degradation [37–40]. Both *sloh3* and *sloh63* mutants appear to promote a response that degrades abnormal proteins under L-heat stress. On the other hand, for *sloh63* specific DEGs, vesicle-mediated transport related to ER stress response and GO terms related to osmotic and salt stress response were enriched (Fig. 7C). As shown in Fig. 5, a significantly enhanced ER stress response was observed in *sloh63* compared to WT and *sloh3*. Furthermore, consistent with the finding that *sloh63* was highly sensitive to salt stress (Figs 2C and 3D), the GO terms for osmotic and salt stress responses were enriched, suggesting that the defect of *SLOH63/MAC17* affects not only L-heat stress but also salt stress responses.

Discussion

Using mutants highly sensitive to L-heat stress, we here isolated the causal genes of *sloh3* and *sloh63* and identified them as *MAC9* and *MAC17*, respectively. Both genes are involved in mRNA splicing. In *sloh3*, a possible nonsynonymous substitution was found in the half-a tetratricopeptide (HAT) domain. This

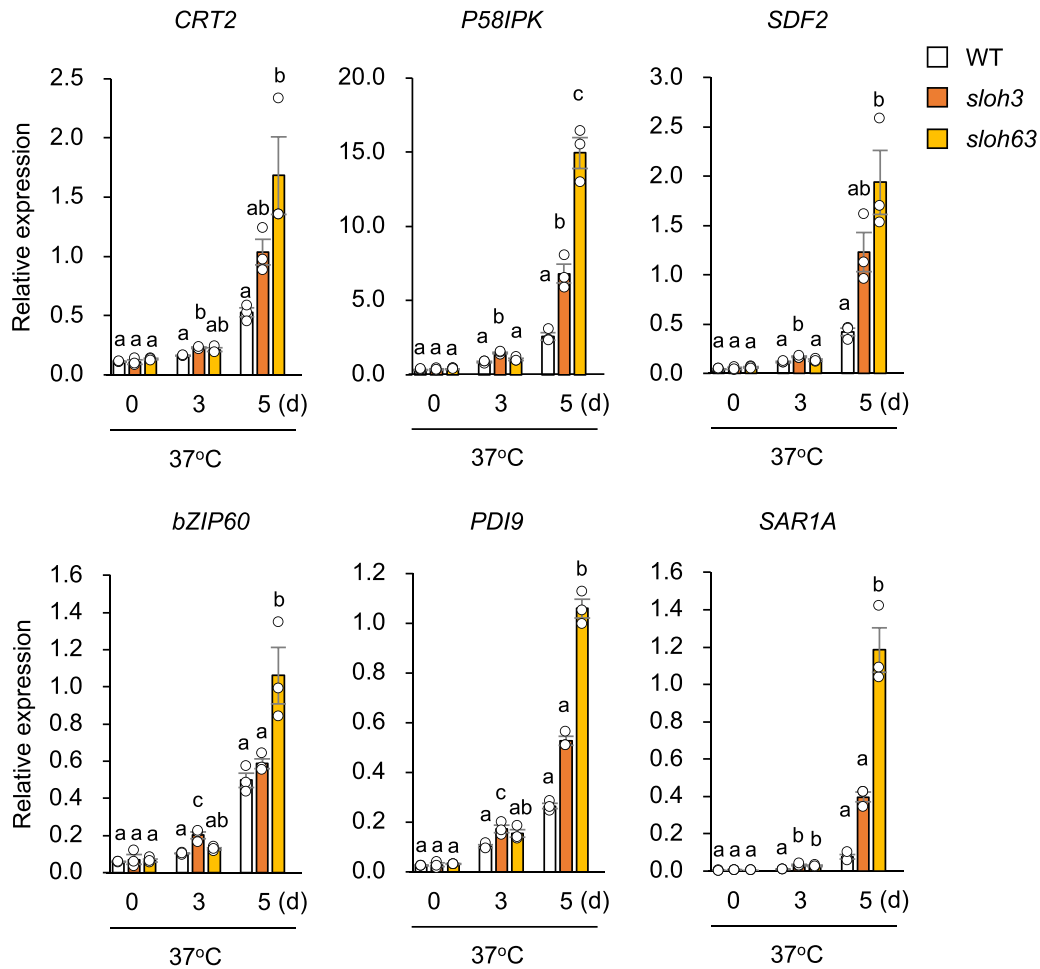


Fig. 5. ER stress responses in *sloh3* or *sloh63* under L-heat stress. Expression levels of genes regulated by bZIP28 (*CRT2*, *P58IPK*, and *SDF2*), genes regulated by bZIP60 (*PDI9* and *SAR1A*) and bZIP60 were measured in 10-day-old wild-type (WT) and mutant seedlings grown at 22°C (0 day) and then placed at 37°C for 3 or 5 days. Data are mean \pm SE, $n = 3$. The same letters above columns indicate no significant difference within a time point in one-way ANOVA with Tukey test, $P < 0.05$.

domain is found in proteins involved in RNA processing and contributes to protein–protein interactions. Mutations in the HAT domain affect RNA processing [41, 42]. The mutation in *SLOH3/MAC9* resulted in the loss of function. In *sloh63*, a possible nonsense mutation in the WD40 domain, which is involved in protein–protein interactions [43], resulted in a truncated protein with one WD40 domain missing. In these mutants, splicing was disturbed and ER stress under L-heat stress was increased; this may explain their L-heat stress sensitivity. These findings suggest that maintenance of appropriate mRNA splicing and suppression of ER stress under L-heat stress are important for stress tolerance.

The *sloh63* mutant, but not the *sloh3* mutant, was significantly more sensitive to salt stress than WT. We have reported that enhanced ER stress impairs not only L-heat tolerance but also salt tolerance [9]. The ER stress was enhanced more in *sloh63* than in *sloh3*, which may impair the salt tolerance of *sloh63*. Both genes encode MAC proteins, which are involved in splicing, and *sloh63* may contribute to splicing that more specifically affects ER stress responses. Alternatively, *sloh63* may contribute to splicing broadly, as it also confers slightly higher sensitivity to L-heat stress than does *sloh3*. In the *sloh63* mutant, root elongation was inhibited. The lack of *MAC5A*, a member of the same MAC protein complex as *SLOH63/MAC17*, inhibits root elongation [44]. The *mac5a*

mutant shows a hypersensitive phenotype in response to a DNA damage inducer, methyl methanesulfonate, suggesting that *MAC5* is important in maintaining the proper response to DNA damage that regulates plant growth and stress adaptation [44]. *SLOH63* may also play an important role in orchestrating growth and adaptation to L-heat and salt stresses.

An increase in the levels of premature mRNAs and expression of ER stress-responsive genes was observed in WT plants under L-heat stress (Figs 4 and 5). Premature mRNA levels and expression of ER-stress marker genes were increased in a PB concentration-dependent manner. Disturbance of splicing such as intron retention caused by L-heat stress may result in the production of structurally abnormal proteins, which in turn may induce ER stress.

We found a single locus responsible for the difference in L-heat tolerance between *Ms-0* and *Col-0*, which we named *Long-term Heat Tolerance1 (LHT1)* (Isono et al., submitted as a back-to-back article). It is identical to *MAC7* and encodes a putative RNA helicase involved in mRNA splicing as a component of MAC. The *mos4-2*, *cdc5-1*, *mac3a mac3b*, and *prl1 prl2* mutants, which are deficient in core components of MAC, are hypersensitive to L-heat stress in comparison with WT, suggesting that MAC proteins are important in L-heat stress responses. Interestingly, *LHT1/MAC7* interacts with *SLOH3/MAC9* and *SLOH63/MAC17* [45]. Both

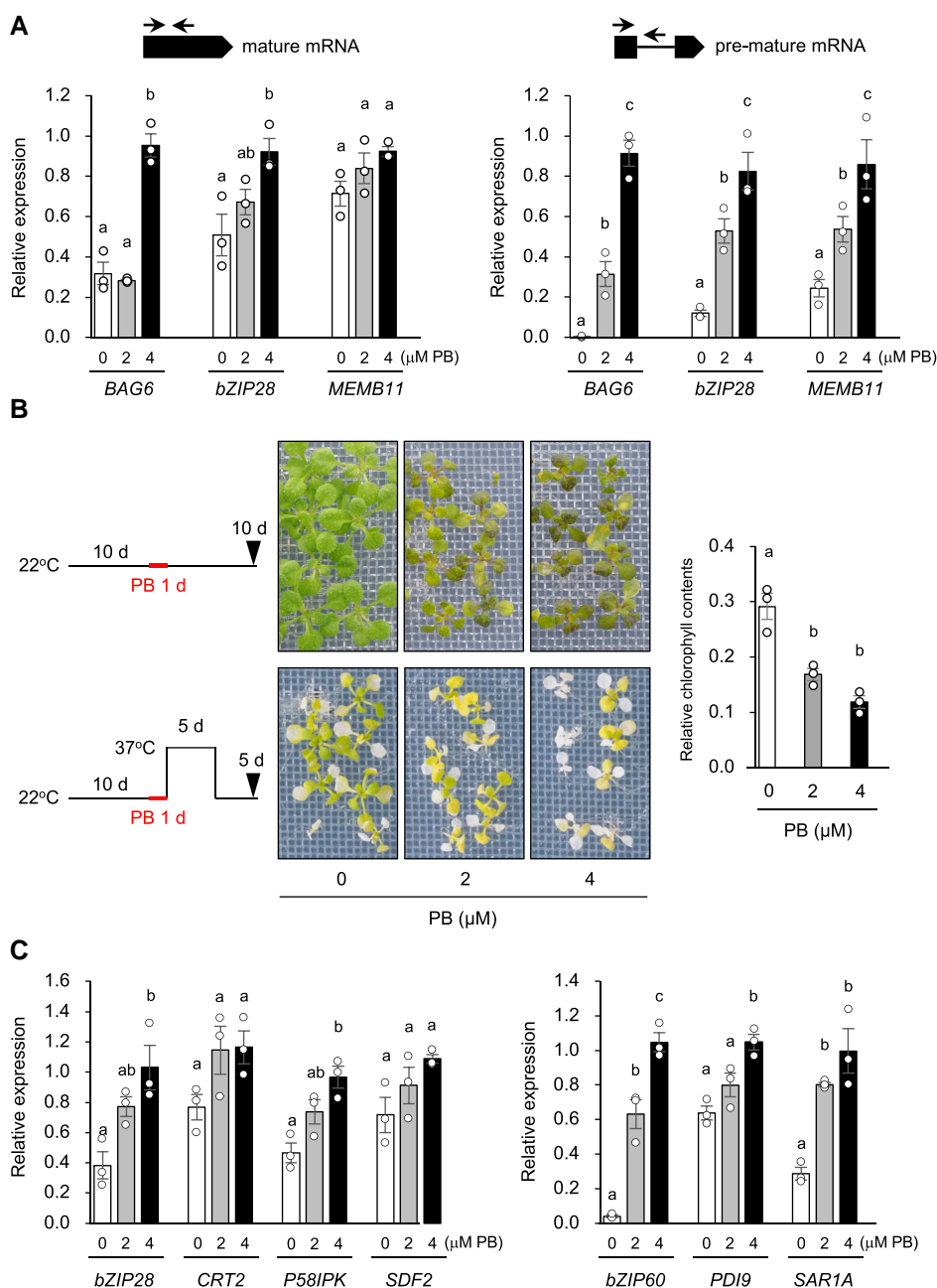


Fig. 6. Effects of splicing inhibitor on alternative splicing, L-heat tolerance, and ER stress response in wild-type (WT) seedlings. **A**) Effects of pladienolide B (PB) treatment on alternative splicing. Expression of mature and premature mRNAs of *BAG6*, *bZIP28*, and *MEMB11* was measured in 10-day-old seedlings grown at 22°C and then mesh-transferred onto water plates containing 2 or 4 μM PB for 1 day at 22°C. **B**) Effects of PB treatment on L-heat tolerance. Seedlings were grown and treated with PB as in (A), subjected to L-heat stress for 5 days, and then grown at 22°C for 5 days; control plants were grown for 10 days at 22°C after PB treatment. Left: plant images. Right: chlorophyll contents relative to those in control seedlings. Data are mean ± SE, *n* = 3. **C**) Effects of PB treatment on ER stress and the following unfolded protein response. Expression levels of genes regulated by *bZIP28* (*CRT2*, *P58IPK*, and *SDF2*) or *bZIP60* (*PDI9* and *SAR1A*) in seedlings grown and treated with PB as in (A). All data are mean ± SE, *n* = 3. The same letters above columns indicate no significant difference within a time point in one-way ANOVA with Tukey test, *P* < 0.05.

quantitative trait locus (QTL) analysis using natural variation in L-heat tolerance (Isono et al., submitted as a back-to-back article) and the forward genetic screening of L-heat-sensitive mutants revealed the importance of proper alternative splicing under L-heat stress in *Arabidopsis*. An LHT1 ortholog, *EMB4*, was also found to contribute to heat tolerance of *Caenorhabditis elegans* (Sato et al., submitted as a back-to-back article). These findings indicate that maintenance of proper splicing under heat stress is a common high-temperature adaptation mechanism in plants and animals.

Materials and methods

Plant materials and growth conditions

Col-0 seeds were sown on plates (90 mm × 20 mm; BIO-BIK, Osaka, Japan) with 1% (w/v) agar containing full-strength Murashige and Skoog (MS) salts, vitamin mixture (10 mg L⁻¹ myoinositol, 200 μg L⁻¹ glycine, 50 μg L⁻¹ nicotinic acid, 50 μg L⁻¹ pyridoxine hydrochloride, 10 μg L⁻¹ thiamine hydrochloride, pH 5.7), and 1% sucrose. The plates were sealed with surgical tape. The seeds were stratified at 4°C for 4–7 days and then transferred to a growth

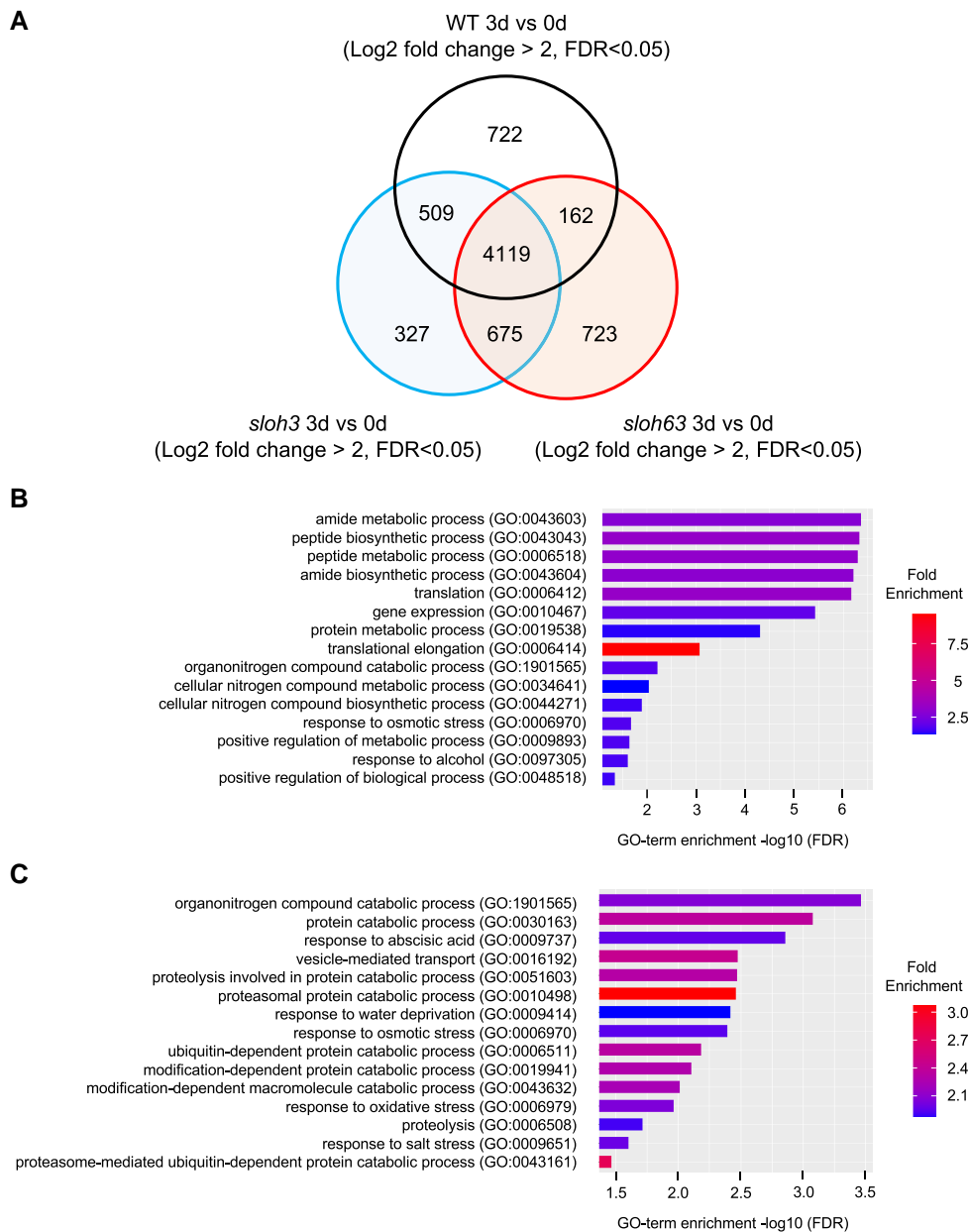


Fig. 7. Effects of L-heat stress on gene expression in *sloh3* and *sloh63*. A) Venn diagrams show the number of differentially expressed genes (DEG) between normal and L-heat stress (37°C for 3 days) conditions (Log₂ fold change > 2, FDR < 0.05) in WT, *sloh3* and *sloh63*. B, C) Applying GO enrichment analysis to 675 common DEGs in *sloh3* and *sloh63* (B) and 723 specific DEGs in *sloh63* (C) at 3 days after heat stress for top 15 biological processes based on positive fold enrichment. The DEGs were classified according to the GO terms in GENEONTOLOGY software (<https://geneontology.org/>) into categories based on biological processes. The colour represents the fold enrichment of the DEGs.

chamber (80 μmol photons m⁻² s⁻¹; 16/8-h light/dark cycle; 22°C) for germination and growth. Seeds of *A. thaliana mac5a* (SALK_132881), *mac11* (SALK_122548), *mac13* (SALK_032761), and *mac14* (SAIL_873_E04) mutants and of the L-heat-stress-tolerant accession Da (1)-12 (CS917) were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University).

Mutagenesis and mutant screening

Seedlings were mutagenized according to the web site <http://cocoabiotech.koko.gov.my/Mutagenesis5.html>. For mutant screening, 10-day-old seedlings grown at 22°C were transferred to 37°C for 5 days and then to 22°C. We isolated candidate mutants on the

basis of their sensitivity to L-heat stress (37°C for 5 days) and selected *sloh3* and *sloh63* for further characterization.

Genetic mapping of the causative genes of *sloh3* and *sloh63*

The *sloh3* and *sloh63* mutants were crossed with Da (1)-12, and the F₁ progeny were self-fertilized to generate F₂ populations. Genomic DNA was prepared from individual F₂ plants with the recessive L-heat-sensitive phenotype for use as PCR templates. The SSLP markers listed in Table S2 were used for mapping. PCR conditions were as follows: 94°C for 2 min; 35 cycles of 94°C for 15 s, 58–60°C (depending on primers) for 15 s, and 72°C for 10 s; and a final 72°C for 2 min. Microsatellites were fractionated in 5–7%

agarose gels, and the recombination value was calculated from the band pattern.

DNA library construction and sequencing

DNA library construction was performed as described in Isono et al. [9].

All libraries were diluted to 10 nM, mixed in equal amounts, and subjected to 1×100-bp single-read sequencing on an Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA). Reads in FASTQ format were generated in bcl2fastq2 Conversion v. 2.18 software (Illumina). The read data were submitted to the DDBJ Read Archive (accession number DRA015832).

Detection of mutations

Mutations were detected as described [14].

Plasmid construction and plant transformation

For complementation tests, the genomic region of At5g28740 or At2g43770 (1 kb upstream of the ATG initiation codon to 1 kb downstream of the terminator) was amplified by PCR with the SLOH3/MAC9 or SLOH63/MAC17 primers (Table S2) and cloned into the pGH35S-mGFP vector. The plant transformation and subsequent selection of transgenic plants were performed as described in Isono et al. [9].

Abiotic stress assays

Seeds were sown on agar plates containing 20 mL MS medium at 22°C. For the L-heat-tolerance assay, plates with 10-day-old seedlings were moved to 37°C for 3 or 5 days and then back to 22°C for 7 days [9]. For the S-heat-tolerance assay, plates with 10-day-old seedlings were placed into a water bath at 42°C for 25 or 35 min and then moved back to 22°C for 7 days. For the S-heat stress after heat acclimation, plates with 10-day-old seedlings were placed at 37°C for 1 h, moved back to 22°C for 2 h, placed into a water bath at 42°C for 150 or 180 min, and moved back to 22°C for 7 days. For the osmotic, NaCl, or LiCl stress assay, 10-day-old seedlings grown on nylon mesh on MS agar plates at 22°C were mesh-transferred to a plate supplemented with 650 mM sorbitol for 21 days; 220 mM NaCl for 7 days or 225 mM NaCl for 11 days; or 30 mM LiCl for 10 days or 40 mM LiCl for 8 days. Chlorophyll content was determined as in [46].

RNA extraction and RT-qPCR or RT-PCR

Total RNA was isolated with a RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and used as a template to synthesize first-strand cDNA with a ReverTra ACE kit (Toyobo, Osaka, Japan). qRT-PCR was performed in a Thermal Cycler Dice Real Time System III (Takara Bio, Shiga, Japan) with Thunderbird SYBR qPCR Mix (Toyobo) in a total volume of 20 µL as follows: 95°C for 30 s, 60°C for 10 s, and 72°C for 10 s; 50 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 30 s; and a final 95°C for 10 s, 65°C for 1 min, and 97°C for 1 s. ACTIN2 was used as an internal standard for qRT-PCR. Primers are listed in Table S2.

PB assay

Seeds were sown on nylon mesh on MS agar plates and placed at 22°C; 10-day-old seedlings grown on nylon mesh on MS agar plates were mesh-transferred to water containing 2 µM or 4 µM pladienolide B dissolved in 0.01% DMSO (Funakoshi, Japan) or 0.01% DMSO (vehicle) for 1 day. Plants were transferred onto MS agar plates, held at 37°C for 5 days, and moved back to 22°C for 5 days.

cDNA library construction and RNA sequencing

Total RNA was isolated with RNAiso reagent (Takara) from 10-day-old seedlings under normal growth (22°C) and heat stress (37°C for 3 days) conditions. All other procedures were performed according to Enoki et al. [47]. The read data were submitted to the DDBJ Read Archive.

GO enrichment analysis

The DEGs between normal and L-heat stress (37°C for 3 days) conditions (Log₂ fold change > 2, FDR < 0.05) in WT, sloh3 and sloh63 were applied to GENEONTOLOGY software (<https://geneontology.org/>) into categories based on biological processes to obtain the GO terms. The obtained GO terms were ordered in descending order for the top 15 biological processes based on positive fold enrichment. These top 15 GO terms are then arranged in descending order of the FDR.

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Supplementary Material

Supplementary material is available at PNAS Nexus online.

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Author contributions

N.E. and T.T. conceived, initiated, and coordinated the project; R.T. and K.I. isolated the sloh3 and sloh63 mutants; N.E. identified the causal genes of these mutants; N.E. performed physiological analyses; A.H. and R.Y. performed the RNAseq analysis; K.T. performed genome sequencing; N.E. detected alternative splicing and ER stress responses under PB treatment; T.T. wrote the manuscript with assistance from I.Y. and Y.S.

Data availability

All data that support the findings of this study are available within its [Supplementary Materials](#).

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